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[INSIGHT IN THE MICROBIAL TECHNOLOGIES AND METHODS 2ND EDITION]

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The falsity of things are as they are because they were as they were.
Adapted from **THOMAS GOLD,**
Astrophysicist

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DEDICATION

THIS WORK IS LOVINGLY DEDICATED TO MY MOTHER

EXECUTIVE SUMMARY

This introductory book provides a grasp on the use of microbial technologies as they function in our everyday lives in developing alternative green technologies based on the intrinsic activity of microbial flora for reducing or even eliminating the products of pollution. Chapter 1 gives us a view on the biocatalytic ability of microbes to diffuse and transform recalcitrant organic sulfur compounds found in fuel. Chapter 2 offers us a description on the bioprocess of converting effluent from the livestock manure waste. Chapter 3 hands us an introduction on using microbial biocatalysts for conversion of waste from natural rubber industry. Chapter 4 describes laccase enzyme production from mycelia cultures of local mushroom species of *Pleurotus florida* (Oyster mushroom) and *Ganoderma lucidum*.

CHAPTER 1

EMERGING MICROBIAL DIBENZOTHIOPHENE DESULFURIZATION FOR FUEL

NOEL M. UNCIANO, & URSELA G. BIGOL

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ABSTRACT

Sulfur emission from fuel contributes to climate change. Our research focus is to isolate microbial biocatalyst using dibenzothiophene as model compound with practical approach to biodesulphurization of petroleum. Isolates were obtained from soil microcosm, culture enrichment with dibenzothiophene (DBT), selection for fluorescence under UV light for the presence of phenol, 2-hydroxybiphenyl (2-HBP), staining using 4-aminoantipyrene and purification using thermal induction. Culture isolates were pre-adapted in diesel by surface growth exposure. UV absorbance scanning was with a recording spectrophotometer to identify 2-HBP spectra of the isolates. Biodesulphurization of commercial feedstock using strain biomass were done in agitated flasks with buffer at 45 ^{o}C. Samples were processed for GC-MS/ or FTIR. One primary isolate, *3Jn* screened positive for phenol, showed similar UV spectra of 2-HBP, the main product of 4S multienzyme pathway for aerobic desulfurization. Biodesulphurization activity depressed by yeast extract appeared to be temperature dependent. The 18 purified, facultative thermo strains were active to both two-ring, benozothiophene (BT) and three-ring, DBT organic sulfur compounds and thus could be widely used as "cleaning" desulfurizing agents. Strains showed differences in "expression" of DBT or BT with opposing orientation, which suggested that activity with organic sulphur compounds was competitive. However, isolates without significant difference in activity were also detected to as much as 67% or greater than 39% among the strains selected. FTIR analyses showed reduction in spectral signals with microbial

treatment. Biodesulphurization based on the optical density of spent media from treated feedstock showed more than 200% activity compared to controls.

THE EMERGENCE OF THE BIOECONOMY

The twenty-first century has been characterized by the emergence of new challenges faced by globalization amidst the need for new socioeconomic and resource scarcity caught by rapid urbanization and population surge, environmental protection and regulation, an expanding global class hungry for automobiles and modern technology, and more volatile finances that face the global market. The term Bioeconomy was the product of these global mosaic of challenges. What Golden & Handfield (2014) had put it, Bioeconomy is global industrial transition of sustainably utilizing renewable aquatic and terrestrial resources in energy, intermediate, and final products for economic, environmental, social, and national security benefits. The White House (2012), declared "bioeconomy is one based on the use of research and innovation in the biological sciences to create economic activity and public benefit." Quoted in the Organization for Economic Co-operation and Development (OECD 2009): "From a broad economic perspective, the bioeconomy refers to the set of economic activities relating to the invention, development, production and use of biological products and processes. If it continues on course, the bioeconomy could make major socioeconomic contributions in OECD and non-OECD countries. These benefits are expected to improve health outcomes, boost the productivity of agriculture and industrial processes, and enhance environmental sustainability."

SIGNIFICANCE

Developing microbial biocatalyst with potential applications at a very low concentration such as occurring at the interface of nanomolar concentration could provide an outright advantage in nanoscale microbial bioremediation. Such specific nano biocatalyst could enhance traits of biocatalytic microbial cells for applications in bioremediation and industrial biorefinery.

OBJECTIVES

To isolate microbial strains for growth on model organic sulfur compound, dibenzothiophene (DBT)

To screen the isolates for biodesulfurization activity using a phenol chromogen, such as 4-aminoantipyrene

To screen the isolates for biodesulfurization activity using UV VIS Scanning Spectrophotometry

To purify the presumptive isolates

To pre-adapt the strains in organic media, suitably diesel

INTRODUCTION

Substantially huge amount of air pollutant is released into the atmosphere when fossil fuels are transformed to energize our society as our world consumes about 85 M barrels of petroleum per day. Sulfur dioxide is a major pollutant with annual emissions of 120 M metric tons of SOx (Grubler 2002) and primarily results to acid rain. Due to its threat of pollution and health hazard, environmental protection law has been legislated in all major regions of the world to require the use of low sulfur fuel. More stringent regulation of 10 to 15 ppm is scheduled for 2011 in Europe and the USA. Preventive measures to limit the release of sulfur dioxide from industries rely on the use of expensive post-combustion technologies (Sharp 2009). Physico-chemical treatment of crude oil using the conventional hydrodesulfurization (HDS) is expensive due to operational cost and reagents such as catalysts and hydrogen. It suffers a technological drawback as it is not proven to efficiently remove organic heterocyclic sulfur components such as dibenzothiophene (Shiflett and Krenzke 2002) (see Figure 1 for the structure) and would incur the use of catalyst volumes of at least four times more for ultra deep desulfurization levels.

Thiophenes remaining in the heavier fractions are resistant to chemical treatment, may comprise about 70% of sulfur compounds in Texas gas oil and 60% of sulfur compounds in Middle East gas oil. Microbial biodesulfurization is being pursued as a potential alternative to "deep desulfurization" to circumvent the use of chemical catalysts. Several authors have proposed Biodesulfurization (BDS) as an alternative technology (Lorenzetti 2001) for removing sulfur from fossil fuels using the biocatalytic properties of cell-free enzymes and intact microbial cells (Monticello 2000, Grossman et al. 2001) to meet the growing demand for ultra clean fuel. Monticello (1996) suggested a multistage process for desulfurization of fossil fuels. This method was based on subjecting vacuum gas oil to HDS prior to BDS in defined conditions. Pacheco (1999) reported that the Energy BioSystems Corporation (EBC) used BDS downstream of HDS. Fang *et al.* (2006) also showed that combination of HDS and BDS could reduce the sulfur content of catalytic diesel oil from 3358 to <20 μg g^{-1}.

For adaptability to a refinery process (distillation and desulfurization), it is desirable that a BDS reaction be carried out at a high temperature, thus the need for thermophilic strains able to grow and desulfurize at high temperature. There are several advantages: higher temperature decreases oil viscosity, makes molecular displacement easier, improves enzymic rates, and decreases bacterial contamination. Several research groups have been working to isolate and characterize thermophilic desulfurizing bacteria and a focus is on improving thermosability of desulfurization enzymes. A *Rhodococcus* enzyme , napthalene dioxygenase has been described with an unfolding melting temperature higher than 95 ^{o}C (Gakhar et al. 2005).

The *Rhodococcus* strains and other mycolic gram positive bacteria have occupied a central role for aerobically removing sulfur from coal and oil in a process termed 4S pathway, an enzyme system producing sulfate and 2-hydroxybiphenyl (which is miscible in the oil phase) without reducing the fuel value. Desulfurization via anaerobiosis produced biphenyl and sulfite, however the yields and rates were very low for industrial application.

The aliphatic chains of mycolic acids (see Fig. 2) along with certain glycolipids help the cells adherence to biphasic phases and ease the dispersion of hydrophobic substances within the contact millieu. They have the capacity to switch the fatty acid composition of their membrane lipids, produce biofilms on ceramic carriers and could thrive under severe conditions. These bacteria could be grouped as K strategists (Margesin 2003) since they can successfully inhabit limited environmental resources and thus remain a dominance in the community. Recent report has described fast-growing strain (Leys et al. 2005) which could effectively bioremediate heavy oil environments. The rhodococci could utilize difficult substrates and have high tolerance with water-miscible and immiscible solvents and may adhere to oil droplets. There have been previous reports that benzothiophene (BT)-desulfurizing gram-positive actinomycetes utilize not only thiophenic compounds but also noncondensed organosulfur compounds such as dimethyl sulfide, dimethyl sulfone, and dimethyl sulfate (Matsui et al. 2000, 2001); thus could also be used for the treatment and disposal of chemical weapons. Bassi et al. (2008) found that a benzothiophene-desulfurizing bacteria, *R. jostii* strain T09, could degrade thiodiglycol (TDG), which is a hydrolyzate of sulfur mustard a chemical weapon.

The substrate specificities of the enzymes involved in desulfurization in various *Rhodococcus* strains were found to be different. *Rhodococcus* sp. K462 can metabolize sulfur from both BT and its alkylated derivatives. Desulfurization of alkylated forms of both dibenzothiophene and benzothiophene by a single strain has also been reported. The strain *R. erythropolis* KA2-5-1 (Tanaka et al., 2002) does not utilize BT but can desulfurize alkylated BT and DBT, while *Rhodococcus* sp. WU-K2R, metabolizes sulfur from BT and from naphthol[2,1-*b*]thiophene(NTH) (Kirimura et al., 2002). DBT and BT utilization genes seemed to be separate since in vitro recombination was needed for expression of both activities (Petrella et al. 2007).

LITERATURE

Microbial biodegradation of organic compounds such as organic sulfur, is important not only in addressing environmental pollution but also in biopharmaceutical, biodiagnostics, and bioindustrial applications. Expression studies involving the utilization/liberation of organic sulfur is scanty although activity related enzymes such as sulfatases have been used for years as diagnostics for the identification of *Mycobacterium* (Tarshis 1965, Mougous et al. 2002) and thus may be important in the diagnostics for Tuberculosis.

The sulfur content of crude oil may vary up to 7.9% (Kilbane and Le Borgne, 2004) and sometimes even up to 14 % (Grossman et al. 1999) and over 200 organic sulfur components have been identified. Thiophenes remaining in the heavier fractions are resistant to chemical treatment, may comprise about 70% of sulfur compounds in Texas gas oil and 60% of sulfur compounds in Middle East gas oil.

Very limited information is available on microbial metabolism of organic sulfur, especially the dibenzo-thiophenes (DBT) found in petroleum products (for recent review please see Kilbane 2006, Mohebali and Ball 2008). Recently, these are the focus of intensive research since their effect on conventional hydrodesulfurization cause a major bottleneck in the refinery industry to reduce sulfur content. The dwindling crude oil scenario has swayed off the balance towards the use of heavy oil reserves, but with a cost. A host of environmental contaminants have to be poured off first before oil could be used by public consumers. These include metals, sulfur, nitrogen, paraffin, polar high molecular weight resins and asphaltenes, which needed advanced technologies of refinery applications. Since hydrodesulfurization (HDS), which is the dominant technology used in refineries for the precombustion desulfurization of fuels would need increased operational and capital costs for the ultra-low sulfur removal of recalcitrant organic sulfur mainly di/benzothiophenes (DBTs) and their alkyl substitutents (Monticello and Finnerty, 1985). There is a need for alternative biotechnologies adapted to the biorefinery/bioprocessing of fuels to be cost effective (with 10 to 15%

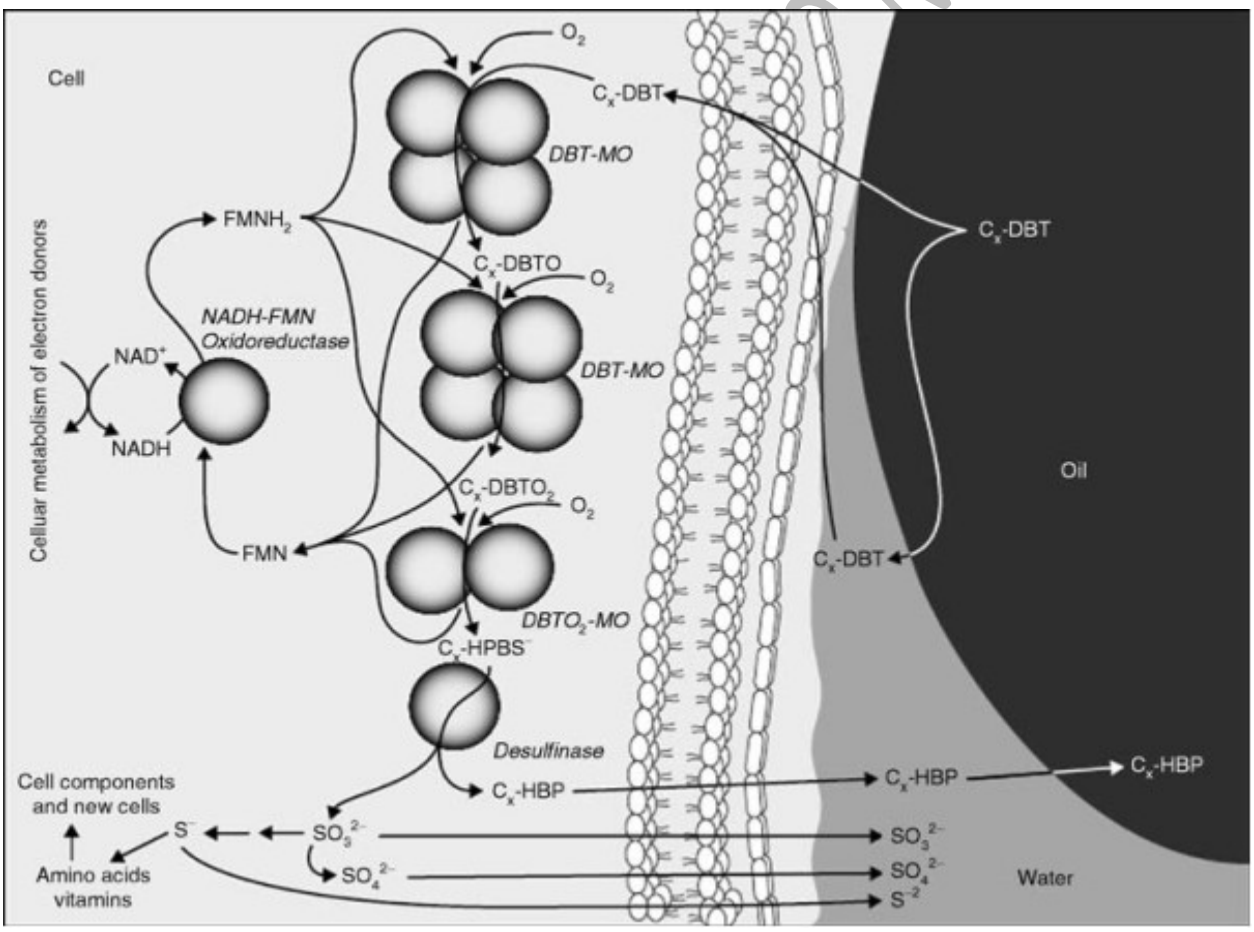
lower cost) and could lessen CO2 emissions, which is estimated to be 70-80% lower (Campos-Martin et al 2010). The applications of microbial biotechnologies could strongly benefit a number of industries in the oil, gas and bioprocessing fields (Kotlar et al. 2004).

As an alternative cell-based biotechnology referred to as biodesulfurization (BDS), the use of the bacterium *Rhodococcus erythropolis* strain IGTS8 or other related microbe results in the selective removal of the sulfur atom from the hydrocarbon skeleton of DBT through a series of enzymes in the 4S oxidative pathway resulting in a conserved hydrocarbon recovery (Kilbane 1992). In the 4S aerobic pathway, DBT is not degraded but only transformed into 2-hydroxybiphenyl (2-HBP, an active fungicide), which partitions to the hydrocarbon phase, while sulfur is eliminated as inorganic sulfate in the aqueous phase containing the biocatalyst. As an advantage thermophilic strains could be used at higher temperatures, resulting to lower viscosity during oil treatment, and a cooling step may be unnecessary.

The pathway was named as 4S and implied consecutive oxidation of DBT sulfur to sulfoxide (DBTO), sulfone (DBTO2), sulfinate (HPBS) and 2-hydroxybiphenyl (2-HBP) as the end product with the release of sulfate/sulfite ions. Several strains of bacteria have been isolated showing the basic metabolic pathway used by *R. erythropolis* IGTS8 including several strains of *Rhodococcus* and related bacteria, *Sphingomonas* and moderate thermophiles *Paenibacillus, Bacillus subtilis,* and *Mycobacterium.* (Ayala and Le Borgne, 2009). The desulfurization genes, dszABC genes are transcribed as an operon, while the dszD gene is unlinked and located in the bacterial chromosome. In certain bacterial genomes as in *Gordonia*, the dsz genes are located in the bacterial chromosome (Gilbert et al. 1998, Rhee et al. 1998).

The dszC gene encodes dibenzothiophene monooxygenase (DszC) that catalyzes the conversion of DBT into DBT sulfone (DBTO2). The dszA gene encodes dibenzothiophene-5,5-dioxide monooxygenase (DszA) that catalyzes the conversion of

DBTO2 into 2-hydroxybiphenyl-2-sulfinate (HBPSi). The dszB gene encodes 2-hydroxybiphenyl-2-sulfinate sulfinolyase (DszB) that catalyzes the conversion of HBPSi into 2-hydroxybiphenyl (2-HBP) and sulfite. The dszABC genes are transcribed as an operon found on a large plasmid, pSOX, in many desulfurization-competent bacteria. An unlinked fourth gene, the dszD gene encoding an NADH–FMN oxidoreductase (DszD), is an accessory component of the desulfurization pathway and allows the regeneration of the cofactors needed for the monooxygenase reactions catalyzed by DszC and DszA. The enzymology of the oxidative desulfurization pathway has been firmly established using purified enzymes from several desulfurization competent bacterial species and from the results of genetic analyses.



Steps in the desulfurization of oil fractions [taken from Monticello, 2000]. Cx-DBT: DBT derivatives; Cx-HBP and Cx-HBPS (HBP and HBPS derivatives produced from Cx-DBT desulfurization); DBTO: DBT sulfoxide; DBTO2: DBT sulfone; DBT-MO, DBT

monooxygenase (DszC); DBTO2 –MO: DBTO2 monooxygenase (DszA); desulfinase (DszB). The corresponding genes are dszC, dszA and dszB in the so called dsz operon (dszABC).

The highly hydrophobic rhodococci cells present an advantageous path for the efficient transfer of hydrophobic DBTs from the oil to the cells. Recombinant solvent-tolerant *Pseudomonas* strains have been used to desulfurize DBT sulfones, which due to their less hydrophobicity are ideal substrates to recover the hydrocarbon skeleton.

These mycolic gram positive bacteria could be grouped as K strategists (Margesin et al. 2003) since they can successfully inhabit limited environmental resources and thus remain a dominance in the community. Recent report has described fast-growing strain (Leys et al. 2005) which could effectively bioremediate heavy oil environments. The rhodococci could utilize difficult substrates and have high tolerance with water-miscible and immiscible solvents and may adhere to oil droplets. There have been previous reports that benzothiophene (BT)-desulfurizing gram-positive actinomycetes utilize not only thiophenic compounds but also noncondensed organosulfur compounds such as dimethyl sulfide, dimethyl sulfone, and dimethyl sulfate (Matsui et al. 2000, 2001); thus could also be used for the treatment of various sulfur compounds. Bassi et al. (2008) found that a benzothiophene-desulfurizing
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Recent applications of nanoscale probes, such as Atomic Force Microscopy (AFM) could reveal insight into the molecular structure of microbes providing clues to their function in bioremediation of environmental contaminants/ pollutants. Studies of Dupres et al. (2009) using high resolution AFM imaging showed the surface of *Corynebacterium glutamicum* as highly ordered with an inner periodic layer presumably of mycolic acids. Using functionalized AFM cantilever tips, Dorobanto et al. (2008) showed that gram positive mycolic acid bacteria such as *Rhodococcus erythropolis* strains have polar hydrophobic surface map suited for attachment to hydrophobic oil droplets.

Another method for the detection of insoluble metal particulates inside and surrounding bacterial cells without distinguishing metal oxidation states is scanning electron microscopy or transmission electron microscopy, followed by energy dispersive X-ray spectroscopy (EDS). Metal elements can be identified using signature spectral lines. Insoluble metal particulates were detected inside and surrounding bacterial cells using scanning electron microscopy or transmission electron microscopy, followed by energy dispersive X-ray spectroscopy (EDS). With this technology, cell-associated precipitates of uranium and technetium in pure culture (Liu et al 2002) and cell-associated uranium

in a microcosm study (Suzuki et al. 2003) could be visualized. EDS can also provide a valuable information about the distribution of the metal-biomass binding throughout the cell structure such as the elemental iron distribution studied with protonated unstained *Sargassum fluitans* biomass (Figueira et al. 1999) as well as the precipitation of nanoscale Ag particles on the surface of the *Geobacter sulfurreducens* (Law et al. 2008).

METHODS

Media and culture conditions. An inorganic sulfur-free medium used for the isolation of microorganisms was a modification of basal salts medium (BSM) of Denome et al. 1994, Abbad-Andaloussi et al. 2003 , with 200 μL vitamin solution, and 5.0 ml mineral solution (Li et al. 2003). The pH was adjusted to ~7 with 10M NaOH and the media was autoclaved at 15 psi for 15 min.

Cultures on solid surfaces (Petri dishes, tube slant cultures) were incubated at room temperature or at 45 ^{o}C. Liquid cultures on a platform environmental shaker were incubated at rpm 180 at room temperature or at 45 ^{o}C as indicated.

Preparation of soil microcosm. Top soil samples from 10 different sites were used in microcosm for the isolation of biodesulfurizing strains. A total of 10 grams of each soil lot placed in 100 ml Pyrex beaker was mixed with 1.5 g of coal and moisten with inorganic-sulfur free medium BSM with dibenzothiophene (DBT). The beakers containing a sample of soil microcosm as described was moisten at least 2 to 3 times a week and maintained at room temperature for several weeks before sampling.

Isolation of desulfurizing strains. 10 grams of microcosm soil was added to 100 ml of BSM with 0.5 mM DBT and shaken for one day. The culture was then made to settle for less than an hour to separate the soil particles. A culture suspension (1:200 inoculum to medium ratio) was added to a fresh BSM medium with 0.5 mM DBT and incubated

while shaken for two days. After the indicated time, a suspension from the latter was used (1:500 inoculum to medium ratio) to inoculate a new set of BSM media with 0.5 mM DBT and incubated for four more days. An aliquot of culture suspensions were plated on the same media containing 1.5% agar to obtain single colonies.

Desulfurization screening assays. Single-colonies obtained in petri dishes were plate-assayed for the identification of strains capable of metabolizing DBT using BSM – agar (1.5%) pre-sprayed with 0.1% DBT solution in ethanol. Selected colonies were then grown in 5-ml BSM with DBT and the culture supernatant were examined for fluorescence under UV light (UV mid-range) using a UV Transilluminator and for the presence of phenolic products.

Detection of desulfurization product. UV light fluorescence was used as presumptive indication for the presence of biphenolic compounds, o,o'-biphenol, OH-Biphenol. The presence of 2-Hydroxybiphenol was also detected with 4-aminoantipyrene (4-AAP) chromogen. A 0.1 ml aliquot of clear fluid from each sample tubes were transferred to 10-ml small test tubes and a 0.01 ml of 5% $K_3Fe(CN)_6$ (in 0.1M pH 9.7 glycine buffer) was mixed into each and followed by the addition of 0.1 ml of 0.25% 4-aminoantipyrene (in 0.1M pH 9.7 glycine buffer). The sample test tubes were examined for unfaded color (reddish).

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UV absorbance spectrophotometry. UV absorbance scanning (200 to 350 nm) was performed with a Shimadzu UV-visible recording spectrophotometer for each of the culture isolates, which were grown in 5-ml BSM (+DBT) or (+BT) for one or two days with control cultures (without DBT, or without BT), and then extracted with an equal volume of chloroform.

Pre-adaptation of isolates in diesel fuel. Culture isolates were pre-adapted in diesel fuel by soaking the media in diesel and streaking the strains on the agar surface. Colonies growing in contact with diesel were isolated.

Correlation of optical density (OD) 600 nm with dry cell weight (DCW). A calibration curve was constructed to correlate OD at 600 nm with dry cell weight. This showed that 1 OD of culture suspension was equated to 0.0204 gram DCW.

Fourier Transform Infrared Spectroscopy (FTIR) or (Gas Chromatography-Mass Spectroscopy GC-MS). Biodesulphurization treatments of commercial feedstock (Bunker/ Crude Oil, Diesel Fuel) using quiescent strain biocatalyst biomass were done in agitated flasks with 120 ml of 0.1 M K-Phosphate buffer ph 7.5 supplemented with 2% glucose at 45 ^{o}C. Biocatalyst incolulum represented about 0.5 ml of 1.2 gram cell paste, which was mixed directly with sample feedstock fuel. Parallel experiments were done with DBT and appropriate controls. Samples were processed for FTIR Attenuated Total Reflectance (ATR) analysis using appropriate conditions.

RESULTS AND DISCUSSION

This study relied on artificial enrichments to isolate strains capable of utilizing model organic sulfur, dibenzothiophene (DBT Figure 1), a generic compound causing sulfur oxide pollution from burnt fossil fuel. This was done by mixing soil samples with this sulfur and coal particles and incubation with moisture. More than 100 isolates (data not shown) were made in slants, which included those initial trials where foul odor formation

was observed. Using a modified media formulated by Scientists at IFP (a French Petroleum Institute), culture specimens were recovered from which one primary isolate, *3Jn* was screened positive, for phenol product formation with a chromogen, 4-aminoantipyrene (see Table 1 below, column 5).

Using another approach, via diesel enrichment in flask cultures, we were able to isolate fluorescent colonies. The photo (Figure 4) was taken from a transilluminated colony plate viewed with a mid-range UV. These isolates (4 2e1, 4 2f1, 4 2f2) produced bright blue fluorescence. However, the fluor is shifted to green with the addition of yeast extract. In a later experiment using a higher concentration of DBT (0.5 Mm in agar slants) with yeast extract, the resulting green fluor was not observed.

These fluorescent strains have the potential in unraveling the molecular topology of the biodesulfurization reaction. Recent work in this aspect by Oldfield *et al.* (1997) and Monticello (2000), showed that the desulfurization reactions occur within the cells. Although data supported an intracellular metabolism of DBT by strain IGTS8, there was no evidence that DBT is actively transported into the cell (Monticello, 2000), and an apparent lack of evidence for mass transfer limitations of DBT (Kilbane & Le Borgne, 2004). It was reported that a major proportion (70%) of the total desulfurization of IGTS8 strain reside in component of the cell envelope (Monticello, 1996).

Fluorescence in response to different media additives is shown Figure 5. Notable was the effect of yeast extract (at 0.2%) in the total and production of green fluor, which was not observed with the addition of ethanol, or casamino acids. This observation was used in establishing a culture medium suited for the maintenance of facultative thermophilic strains.

Biodesulfurization activity of isolates was affected by yeast extract. In Table 2, it was shown that this greatly depressed the cells activity, which caused the disapperance of the 262-267 nm peak at ambient temperature and at 45 ^{o}C. The 239 nm peak was also

reduced much more at room temperature (a difference of 11.6%). Since the isolates were less sensitive to yeast extract at a higher temperature of 45 ^{o}C, it was then practical to maintain these strains at the higher temperature.

Comparative study (Table 3) on the activities of primary isolate *3Jn* revealed that there was a relative tendency in the reduction of both peaks at a higher temperature after pre-adaption. In a separate experiment using 3 isolates (which had been partially purified), 1RTa, 1Rtb, & 1 c1, again it was observed that pre-adapted cultures had lower activities at both peaks, especially with the 268 nm peak (Table 4). However, the 239 nm peak was relatively not affected, except for isolate 1 c1.

As an alternative low-cost or "cheap" substrate to DBT, dimethylsulfoxide (DMSO) could be used instead, for growth expanding of the cultures (Please see Figure 5).

Tables 5 and 6 show that the isolates are active to both dibenzothiophene (DBT) a three ring organic sulfur and benzothiophene (BT) a 2-ring organic sulfur. These isolates could be grouped into at least three according to the level of 267 absorbance. The highlighted isolates showed both high activities of 267 and 239 nm peaks.

Table 7 showed the absorbance of the major spectral peaks, which indicated the DBT desulfurization activities of the microbial isolates before pre-adaptation with diesel fuel. These two spectral peaks (one broad peak with a range of 271 to 262 nm and the other peak at about 239 nm) characterized the biodesulfurization activities of the microbial isolates. After pre-adaptation with diesel (please see Tables 8 & 9) the broad peak has narrowed with the elimination of 271 and 262 nm peaks. On the other hand, the activity at 239 nm was not significantly affected. A summary table (Table 10) showed a comparison of the broad peak before and after pre-adaptation with diesel. The molecular polishing of the peak absorbance was consistently observed with the isolates.

Microbial treatment of model organic sulfur, DBT showed a near stoichiometric conversion (Figures 10 & 11) of DBT, (which was the only UV active substrate present in the medium) most probably via the enzymatic 4S pathway resulting in the production of 2-HBP (2-hydroxybiphenyl).

The strains showed differences in "expression" of DBT or BT with opposing orientation (direction), which suggested that activity with organic sulphur compounds was competitive. However, isolates without significant difference in activity were also detected to as much as 67% or greater than 39% among the strains selected (Please see the Table: Expression Profile of Strains, data shaded in green showed no significant difference (<20%).

Attached figures of FTIR analyses showed convincing results indicating a clear difference in spectral signals of microbial treated commercial feedstock fuel, especially with diesel, which significantly had lower spectral intensity compared with non-treated diesel (Figure 9). Dignostic regions (major spectral peaks) were not affected by microbial processing and indicated that the calorific value (aliphatic residues) of the fuel is conserved. Crude Oil fractions treated with the isolated strains revealed enhanced transmittance [%T] in FTIR indicative of a clearing action and had a marked decrease in viscosity. Using resting microbial cells, the biodesulfurization activity (OD) in the spent media of treated crude oil feedstock showed more than twice (2X) the activity compared to controls (Figure 13). Thus, the strain exhibited biocracking property by releasing soluble by products in the aqueous phase. Further, a "light oil" fraction was also produced from crude oil making this process a highly efficient direct microbial catalytic bioconversion (Please refer to the pathway scheme Figure 14).

CONCLUSION AND RECOMMENDATION

This study supported published literature on biodesulfurization using various microbial strains. There is a clear indication that the strains produce 2-Hydroxybiphenyl, which is

the main product of 4S multi-enzyme pathway for aerobic desulfurization. This was shown by the alignment of spectra of the cultures with pure standard of 2-Hydroxybiphenyl and by their sensitivity to yeast extract. The 18 purified facultative thermo strains, have the potential due to their activities to both two-ring and three-ring organic sulfur compounds. This feature seemed to be rare since published literature showed that in vitro recombination was needed for co-expression. These partially purified strains as well as the primary strains and fluorescence-associated strains represent our most recent addition to the ever increasing resources suited for biodesulfurization. Induced phenotypes may be forthcoming with strains adapted for growth with organic compounds.

To be able to verify our results, ion-chromatography and mass spectroscopy of the samples are recommended especially to be able to distinguish the molecular species of the peaks 267 and 239 nm. Furthermore, flask cultures will be done with crude oil/ bunker oil to ascertain our claim that the strains are indeed capable of application in the industry.

SOCIO-ECONOMIC IMPACT

In highly populated regions such as China, the transport sector contributed 70% to 80% of air pollution. More than billions of people are exposed to polluted air (WHO), causing millions of premature deaths. The cost of air pollution based on the estimates of the World Bank amounted to about 2% of GDP in developed countries or 5% of GDP in developing countries. Low sulfur transport fuel could result not only in lower sulfur emissions but could also assist in lowering NOx, HC (hydrocarbons), CO (carbon monoxide), PM (particulate matter). Management and recycling of spent catalysts are the major problems of the petroleum refinery industry, which could also be benefitted by using a microbial based-biodesulfurization process.

The world generation of the spent metal-bearing, hydrotreating (HT) catalyst was estimated (Dakota Catalyst, 1996) to be about 87,500 t/y and spent desulphurization

catalyst at 297,500 t/y from the petroleum refinery industry. Rao (1993) estimated that there were about 336 FCC (Fluid Catalytic Cracking) units operating around the world, each processing 3,300 t/d of feed and requiring 2-3/t/d of fresh catalyst make up. This gives a worldwide total spent FCC catalyst between 250,000 to 368,000 t/y.

TARGET BENEFICIARIES

Small as well as medium scale biorefineries could use microbial biodesulfurization technology in a cost effective production scheme.

The petroleum refineries would likewise benefit using a microbial mediated recovery/recycling of spent catalysts. Hydro-desulfurization (HDS/RDS) of heavy oil produces spent catalysts that contain molybdenum (Mo), vanadium (V), nickel (Ni) or cobalt (Co) at concentration levels that has been found to be economical for recovery. The energy savings and environment benefits associated with these recycling activities are also quite significant. It has been estimated that recycling of various metal scraps consumes approximately 33% less energy and generates 60% less pollutants than the production of virgin material from ore. Spent catalysts are truly a valuable source of metals as they contain up to 10% molybdenum and/or vanadium, 3% nickel or cobalt and 50% alumina.

EXPECTED OUTPUT

Production of specific microbial biocatalyst to be used for fuel biodesulfurization. Describe microbial strains applicable for the recovery, recycling of spent catalysts from the petroleum industry.

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FURTHER RESEARCH SCHEME & ACTIVITIES AT A GLANCE

Innovative Queue	Activity	Experimental	YR 1	YR 2	Remarks	
Culture Propagation	Sourcing of screened biodesulfurization isolates which were obtained from microcosm	8 to 10 isolates, propagated in culture slants	Q 1		Propagated at the EBD - ITDI Laboratory	
	Propagation and up-scale culture	8 to 10 isolates, growth culture-flasks	Q 1			
Genotypic Screen of the Isolates	Genomic screen using Randomly Amplified Polymorphic DNA (RAPD)-PCR for the biodesulfurization dsz ABC gene cluster	PCR gene primer sets: A) Duarte et al. 2001. dszA,B,C (w/ forward & reverse) B)Bustos-Jaimes et al.2003. dszC1,dszC3,		Q 2 to Q 4	Gene sequencing services with Macrogen, Korea	

		dszA2,dszC3 Three replicates for each				
	Expression of the dsz ABC genes using Quantitative RT-PCR	PCR gene primer sets: dsz A,B,C Three replicates for each		Q 2 to Q 4		
	Ribotyping using the 16S rDNA sequence analyses	PCR 16S rDNA primers at bp 8, and at bp 536 Three replicates for each	Q 2 to Q 4		Gene sequencing services with Macrogen, Korea	
	Mycolic Membrane fatty acid analyses (FAME)	7. to 10 strains Representative sample	Q 2 to Q 4		Option to outsource with laboratories abroad	
Cell Surface Imaging of Bacterial Strains	Preparation of culture samples for Scanning Electron Microscopy (SEM)	Representative samples	Q 1			
	Preparation of Culture samples for Atomic Force Microscopy (AFM)	Representative samples			AFM made available by First Sem 2011 MBB UP Dil	
Induced Biometal stress on	Preparation of Culture samples for SEM			Q 1 /&		

Bacterial Strains				Q 4		
	Preparation of Culture samples for AFM	Biometallic ions: mercury, aluminum, arsenic, molybdenum, nickel, cobalt, iron: To use three different concentrations				

OBJECTIVES

Input into processes for bioremediation of industrial pollutants/contaminants is of great importance to address critical issues as society becomes more exposed to environmental risks (resulting from processes leading to climate change). However, only a few studies deal on the understanding of basic mechanisms involved in microbial remediation, such as how microbial cells interact with a particular chemical pollutant in association with a myriad of complex constituents as affected by environmental-physical conditions occurring in contact with microbial cells. Delineating these processes will need nanoprobes such as Atomic Force Microscopy and Scanning Electron Microscopy to assess microbial surfaces in association with particular chemical species such as metal ions. Digital graphics of these interaction could give information as to how decontamination, bioremediation of toxic pollutants could be improved. Specific traits of biocatalytic microbial cells are stored in the genomic make-up of these microorganisms and there is a need to be able to detect for the presence of specific enzymes capable of converting the organic sulfur into less toxic substances. Quantitative PCR will likewise be used to assess the expression state of the biodesulfurization (dsz) genes present in the different isolates. Using 16S ribosomal

genes probes, we will be able to assign to which taxonomic groups, our microbial isolates belong. This will help us compare the effectivity of the isolates with other previously characterized strains. Thus, the project will provide information about microbial cell surfaces as affected by various environmental-physical conditions, especially metal ions and assess as to whether such traits are associated with the type and genomic constituents of the particular microbial strain.

PROCEDURE/METHODOLOGY

Chemicals. All chemicals were of analytical grade and obtained from local distributors. Trace metals were chloride salts and $MgSO_4$ was omitted since sulfate was found to inhibit the biodesulfurization. Organic sulfur, dibenzothiophene (DBT) was prepared aseptically as 0.5 M (1000x) solution in N'N'-dimethylformamide.

Equipments. The following instruments were used in this study, namely: Orbital shaker incubator (YIH DER LM-590R), pH meter, digital (JENCO 1671), autoclave, UV VIS Scanning Spectrophotometer (Shimadzu/ Thermo), VWR PCR Thermocycler, Quantitative RT-PCR.

Source of bacteria

The biodesulfurization isolates are maintained at the EBD laboratory and were previously purified from the previous project at ITDI.

Media and culture conditions

An inorganic sulfur-free medium used for the isolation of microorganisms was a modification of basal salts medium (BSM) of Denome et al. 1994, Abbad-Andaloussi et al. 2003 , with 5.0 ml mineral solution (Li et al. 2003) and 0.2% yeast extract. The maintenance medium is a modification of the basal salts medium (BSM) described above, which in addition contained 0.5mM dibenzothiophen (DBT) and 4,6 dimethyl

dibenzothiophene (4,6 DMDBT). The pH was adjusted to ~7 with 10M NaOH and the media is autoclaved at 15 psi for 15 min.

Cultures on solid surfaces (Petri dishes, tube slant cultures) are incubated at room temperature or at 45 ^{o}C. Liquid cultures on a platform environmental shaker are incubated at rpm 180 at room temperature or at 45 ^{o}C as indicated.

Identification of the isolates

Preliminary characterization of bacterial strains will be performed by Scanning Electron Microscopy and staining. Fatty acid analyses will be outsourced with Microbial ID Inc. (Newark, DE, USA) or by other suitable means or alternatively profiled as described below.

Profiling of major membrane fatty acids of the isolated strains

The fatty acid profiling of the strains are to be performed using the protocol described by Kaushik (2002). The chromatography is accomplished using a gas chromatograph with mass spectrometer. A PE-Elite-5 capillary column (ID 0·32 mm, length 15 m and 0·25 Mm film thickness) is used with helium as the carrier gas. The oven temperature is initially kept at 80°C and is increased to 300°C at a rate of 5°C min^{-1}, with a final holding time of 20 min at 300°C. The mass spectrometer is operated at 70 eV of electron ionization energy. The injector and detector temperatures are set at 200 and 300°C respectively. The fatty acid profiling is to be carried out by comparison of the chromatograms with various mass spectral libraries as well as with chromatograms of authentic standards.

Genomic DNA extraction and 16S rDNA PCR

Polymerase Chain Reaction (PCR) nucleotide probes of the 16S ribosomal genes will be used to test for the taxonomic group of the microbial strains. The strains will be identified by 16S rDNA sequence analyses. Both strands of the first 523 bases of the 16S rRNA gene will be sequenced by outsourced laboratories, such as in Korea

(Macrogen) after PCR using primers that anneal at the positions beginning at base pair 8, 5P AGAGTTTGATCITGGCTCAG-3P, and 536, 5P-GTATTACCGCGGCTGCTGGCAC-3P, in the 16S rDNA gene of *E. coli*.

Genomic DNA is isolated from 15 ml of a suspension of cells cultivated in Minimal media supplemented with 1.5 mM DBT (and harvested during the stationary phase) by using GenElute™ bacteria genomic DNA kit with a cetyl trimethylammonium bromide method. Bacteria are incubated at 37°C for 1 h with 375 U of mutanolysin before adding lysozyme to improve cell lysis. DNA concentration and purification are determined spectrophotometrically by A260 and A260/A280 calculation, respectively.

16S rDNA primers

based on16S rDNA gene of E. coli

sequence beginning at base pair 8:

5'-AGAGTTTGATCITGGCTCAG-3'

sequence beginning at base pair 536:

5'-GTATTACCGCGGCTGCTGGCAC-3'

The PCR mixtures are prepared (Duarte et al. 2001) with 1 µl of target DNA (ca. 10 to 20 ng), 5 µl of 10x Stoffel buffer (10 mM Tris-HCl [pH 8.3], 10 mM KCl), 200 µmol of each deoxyribonucleoside triphosphate, 3.75 mM MgCl2, 20 pmol of each of the appropriate primers, 1% (vol/vol) formamide, 5 U of Taq DNA polymerase, and the Stoffel fragment (Perkin- Elmer/Cetus) in a 50-µl final volume. The reaction mixtures are overlaid with mineral oil (Sigma, Zwijndrecht, The Netherlands). A hot-start procedure (5 min, 94°C) is used before the enzyme was added to prevent aspecific annealing of the primers (4). Negative controls (PCR mixture without added target DNA) are included in all PCRs.

Amplification is performed with the Taq polymerase (Qiagen or Amersham Pharmacia Biotech) according to the manufacturer's recommendations.

The PCR conditions are as follows:

– one cycle of 1 min at 95°C;

– 30 cycles consisting of 1 min at 95°C, 1 min at 60°C and

1.5 or 4 min at 72°C;

– and a final cycle of 10 min at 72°C.

Both strands of the first 523 bases of the 16S rRNA gene PCR products are to be sequenced (Macrogen Labs, Korea). While the sequence comparisons between the PCR products and the 16S rDNA gene are performed with the BLAST programs of the National Center for Biotechnology Information (NCBI).

RAPD Analysis and PCR Amplification of the *dsz ABC gene cluster*

Polymerase Chain Reaction (PCR) probes from published papers will also be used to detect for the presence of particular enzymes such as the 4S pathway comprising the dsz ABCD genes. This will provide information on which enzymes are active in the organic sulfur bioremediation pathway.

PCR primers alternative sets:

A. from Duarte et al. 2001

			expected product
dszA	forward	5'-TCGATCAGTTGTCAGGGG-3'	547 bp
	reverse	3'-GGATGGACCAGACTGTTGAG-5'	
dszB	forward	5'-ATCGAACTCGACGTCCTCAG-3'	422 bp
	reverse	3'-GGAACATCGACACCAGGACT-5'	
dszC	forward	5'-CTGTTCGGATACCACCTCAC-3'	392 bp
	reverse	3'-ACGTTGTGGAAGTCCGTG-5'	

B. from Bustos-Jaimes et al. 2003

dszC1 5'-CGCGAATTCATGACACTGTCACCTGAAAAGCAGC-3'
(dszC start codon underlined)
dszC3 5'-CCCAAGCTTCTCAGATCCTCAGGAGGTGAAGCCGGG-3'
(dszC stop codon underlined)

For dsz operon

dszA2 5'-CGCGAATTCAGGACGCATACGCGATGACTCAACAAC-3'
(dszA start codon underlined)
dszC3 5'-CCCAAGCTTCTCAGATCCTCAGGAGGTGAAGCCGGG-3'
(dszC stop codon underlined)

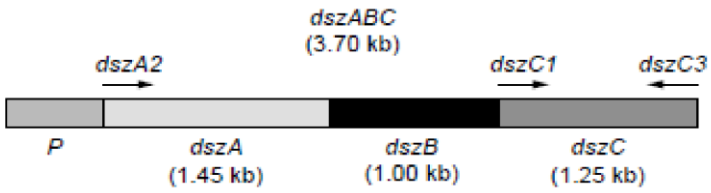

Figure 2
Organization of the *dszABC* operon of *R. erythropolis* ITGS8.

PCR with the dszB and dszC detection systems entailed 35 cycles (94°C for 1 min, 55°C for 1.5 min, 72°C for 2 min). For the dszA detection system, a touchdown PCR (9, 36) was performed, with the annealing temperature decreasing by 1°C every second cycle, from 65 to 55°C, with 15 additional cycles done at 55°C. A final extension was carried out at 72°C (10 min). Primers used for the dszA, dszB, and dszC systems were based on the sequences of the *R. erythropolis* strain IGTS8 dsz gene cluster. Primers were designed (Duarte et al. 2001) using the PRIMER program of the CAMMSA suite at the CAOS/CAMM center (University of Nijmegen, Nijmegen, The Netherlands). The criterion used for primer selection was the absence of adventitious amplification of any nontarget sequences. A search of the EMBL database using FastA did not reveal

significant homologies to sequences other than those of the dsz gene regions, which validated the use of the dszA, dszB, and dszC PCR systems for direct screening. These primer sets are specific for the respective dsz region of strain IGTS8, as they only produced amplicons of the expected sizes with genomic DNA of this strain, not with genomic DNA of a suite of 25 other bacterial species (Duarte et al. 2001).

Ready-To-Go RAPD™ Analysis Beads (GE Amersham) have been optimized for RAPD reactions and contain thermostable polymerases (AmpliTa q™ DNA polymerase and Stoffel fragment), dNTPs (0.4 mM each dNTP in a 25 µl reaction volume), BSA (2.5 µg) and buffer [3 mM MgCl2, 30 mM KCl and 10 mM Tris, (pH 8.3) in a 25 µl reaction volume]. The two different thermostable polymerases, combined in a proprietary ratio, produces a more complex RAPD fingerprinting pattern than either of the polymerases alone. The only re a g e n t s which must be added to the reaction are an arbitrary primer and template DNA. The Ready-To-Go bead format significantly reduces the number of pipetting steps, thereby increasing the reproducibility of the RAPD technique and minimizing the risk of contamination.

RAPD primers should at a minimum be purified using a NAP™-10 column (17-0854-01, -02). Their GC content should be at least 60%, and they should contain no hairpin structures.

Procedure A: RAPD Reaction

When performing RAPD analysis, exercise extreme care to prevent DNA contamination. Always use sterile, filter pipette tips and avoid carry-over contamination of stock solutions.

• Check that the bead is visible in the bottom of the tube of R A P D Analysis Beads. If necessary, tap the tube against a hard surface to force the bead to the bottom of the tube.

• Add the following to a tube containing the RAPD Analysis

Bead :
25 pmol of a single RAPD primer X µl
5-50 ng template DNA Y µl
Distilled water to total of 25 µl

• Mix the contents of the tube by gently vortexing, or by repeatedly pipetting the mixture up and down. Centrifuge briefly to collect the contents at the bottom of the tube.

• Overlay the reaction with 50 µl of mineral oil.

• Place the samples in a thermocycler and cycle using the following cycle profile: 1 cycle at 95°C for 5 minutes followed by 45 cycles at 95°C for 1 minute, 36°C for 1 minute and 72°C for 2 minute.
Amplification was performed with the Taq polymerase *(Amersham Pharmacia Biotech)* according to the manufacturer's recommendations.
The PCR conditions were as follows (Bustos-Jaimes et al. 2003):
– one cycle of 1 min at 95°C;
– 30 cycles consisting of 1 min at 95°C, 1 min at 60°C and 1.5 (for *dszC*) or 4 min (for *dsz*) at 72°C;
– and a final cycle of 10 min at 72°C.

• Continue with Pro c e d u re B, Gel Analysis.
Procedure B: Gel Analysis
After amplification, the banding pattern of the randomly amplified DNA must be visualized and analyzed. RAPD analysis can be done on either agarose or polyacrylamide gels. For agarose gels:

• Pour a long (e.g. GNA 200) 2% agarose gel using 1X TAE or TBE

b u ffer (see Appendix 1) containing 0.5 µg/ml of ethidium bromide [or red gel dye].

• Add 1 µl of 6X tracking buffer (see Appendix 1) to 5 µl of the amplified sample and load onto the gel.

• Electrophorese the sample until good separation of RAPD bands is observed and the bromophenol blue from the tracking dye is 2.5 cm or less from the bottom of the gel (e.g. 150 volts for 3 hours).

For better resolution of low molecular weight bands, polyacry l a - mide gel systems may be used. The loading level for a p o l y a c rylamide gel should be approximately 0.5 µl of the 25 µl RAPD reaction. For visualization on acrylamide gels, we recommend using the DNA Silver Staining Kit from Amersham Pharmacia Biotech.

Note :Bands and/or smears in a “no template” control are normal in RAPD Analysis. See page 21 for further information.

Total DNA from *Rhodococcus erythropolis* IGTS8 and *E. coli* BL21 or a suitable control specimens are to be used as PCR templates for positive and negative controls, respectively. Agarose gel electrophoresis of PCR products is performed in 0.8% agarose gels in Tris–Borate–EDTA (TBE) buffer at 75 V for 90 min (Sambrook et al., 1989).

The sequences of both strands are sequenced (Macrogen Labs, Korea) and sequence comparisons between the PCR products and *R. erythropolis* IGTS8 dsz genes were performed with the BLAST programs of the National Center for Biotechnology Information (NCBI).

Preparation of culture samples for biometallic stress interaction

Each of the culture isolates, which are grown in 5-ml BSM or in flask cultures with (+DBT) or (+BT) or (+4,6 DMDBT) for one or two days with control cultures without the relevant organic sulfur substrates, are then subjected to different concentrations of biometallic ions such as mercury, arsenic, aluminum, molybdenum, vanadium, nickel, iron or cobalt, and sampled cultures for AFM analyses and SEM imaging.

Preparation of culture samples for Atomic Force Microscopy (AFM) analyses

The cells are harvested by centrifugation at 4500 x g for 5 minutes at 4 C and washed twice in phosphate buffer (pH 7.4). After that the cells are fixed in PBS containing 2.5% glutaraldehyde for 2.5 h at 4 C. Fixed cells are washed and resuspended in phosphate buffer. Alternatively the cells suspension are simply dried in air. About 2 μL of cell suspension is placed in a clean [cover slip] slide and examined with atomic force microscope (NASAT Corp., or with Dr. C. Saloma MBB and National Institute of Physics).

Cell Immobilization for AFM Measurements

Applying AFM to the examination of living microbial cells requires a robust technique for cell immobilization, while avoiding denaturation. Effective immobilization techniques must position the cells such that they are firmly attached to a support and stable to tip forces in liquid environments that favor viability. Species of bacteria in this study are bound to the surface of glass slides coated with 3-aminopropyltrimethoxysilane or fixed using 2.5% glutaraldehyde. A droplet of concentrated bacterial suspension (5-10 μL) is placed onto a silanized glass slide. After 60 min of settling, the bacteria-coated glass was rinsed to remove loosely attached cells and transferred to the AFM stage. Slides are kept hydrated the entire time prior to AFM work by soaking the slide in phosphate buffer. All measurements are made at room temperature under a 0.1 M phosphate buffer solution.

The AFM microscope is equipped with a scanner that had a maximum X–Y scan range of 100 × 100 μm, at a Z-range of 7 μm, which is operated by means of a real-time closed loop scanning control system that allows for the accurate measurement, repositioning and zooming in on selected features (ScanMaster; Park Scientific Instruments). The scanner is calibrated in the three directions by means of a VLSI reference standard (VLSI Standard, San José, CA, USA). The images are acquired using silicon cantilevers with high aspect ratio conical silicon tips (Ultralevers; Park Scientific Instruments). The force constants are 0.03 N/m for contact mode imaging and 7.4 N/m for intermittent contact mode imaging. In order to be able to locate the area of interest on the samples and identify any damaged bacteria, we used the built-in long-range on-axis microscope, which is capable of a 5:1 zoom and ×3500 magnification. Intermittent contact mode imaging, which makes it possible to use higher scan rates and which can cope more easily with steep features, was used to acquire images of whole bacteria at scan speeds between 5 and 50 μm/s. Contact mode imaging used at scanning speeds between 1 and 10 μm/s gave less satisfying results due to strong adhesion phenomena between tip and sample, giving rise to image blurring. Accurate feedback tuning was necessary in both imaging modes in order to obtain the maximum possible gain that allowed the resolution of bacteria surface structures while avoiding oscillations when scanning along the side walls of the cell. All images were acquired as 512 × 512 pixels, and processed by means of plane-fitting, high-frequency filtering and three-dimensional shaded rendering. Cross-sections of interesting features were obtained by using the image analysis software of the microscope to acquire numerical topographical information. A typical imaging session began by using the built-in optical microscope and moving the X–Y table in search of bacteria showing signs of damage. The AFM cantilever was then moved toward the surface in the proximity of the chosen bacterium. A large scan (50 × 50 μm) was made in order to assess the exact position and nature of the bacterium, with further smaller scans being used to zoom in on any interesting features.

Using nanoprobes of Atomic Force Microscopy (AFM) it will be possible to assess digital images of microbial cells to provide surface topologies or characteristics, which could provide insight into possible mechanistic effect of physico-chemical parameters in association with particular chemical species such as metal ions.

Preparation of culture samples for Scanning Electron Microscopy (SEM) or SEM-Energy-Dispersive X-ray Spectroscopy (EDS)

Cultures were grown under the conditions described above and separated from culture media by centrifugation at [13,000 x g for 5 min] or [4500 x g for 5 minutes] then dried under vacuum. The resulting material was applied to carbon tape on a sample stage and analyzed on a scanning electron microscope equipped with an x-ray detector. The sample was subjected to 20 keV incident energy and measurements were made over a 50 s interval.

Scanning Electron Microscopy (SEM) will be used to assess surface morphology of microbial cells as they are subjected to various conditions in the presence of select chemical species such as metal ions.

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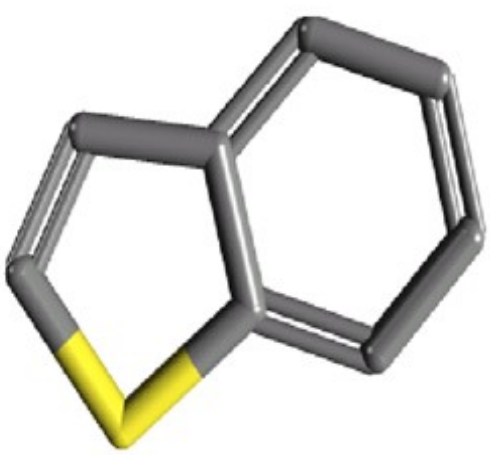
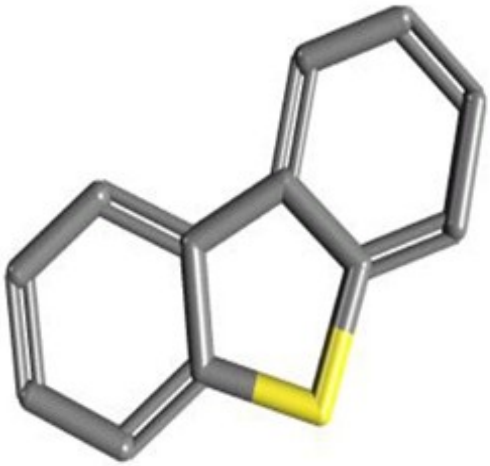
FIGURES AND TABLES



Benzothiophene (BT)



Dibenzothiophene (DBT)

MODEL ORGANIC SULFUR COMPOUNDS RESISTANT TO HYDRODESULFURIZATION (HDS)

Figure 1. Refractory organic sulfur compounds.

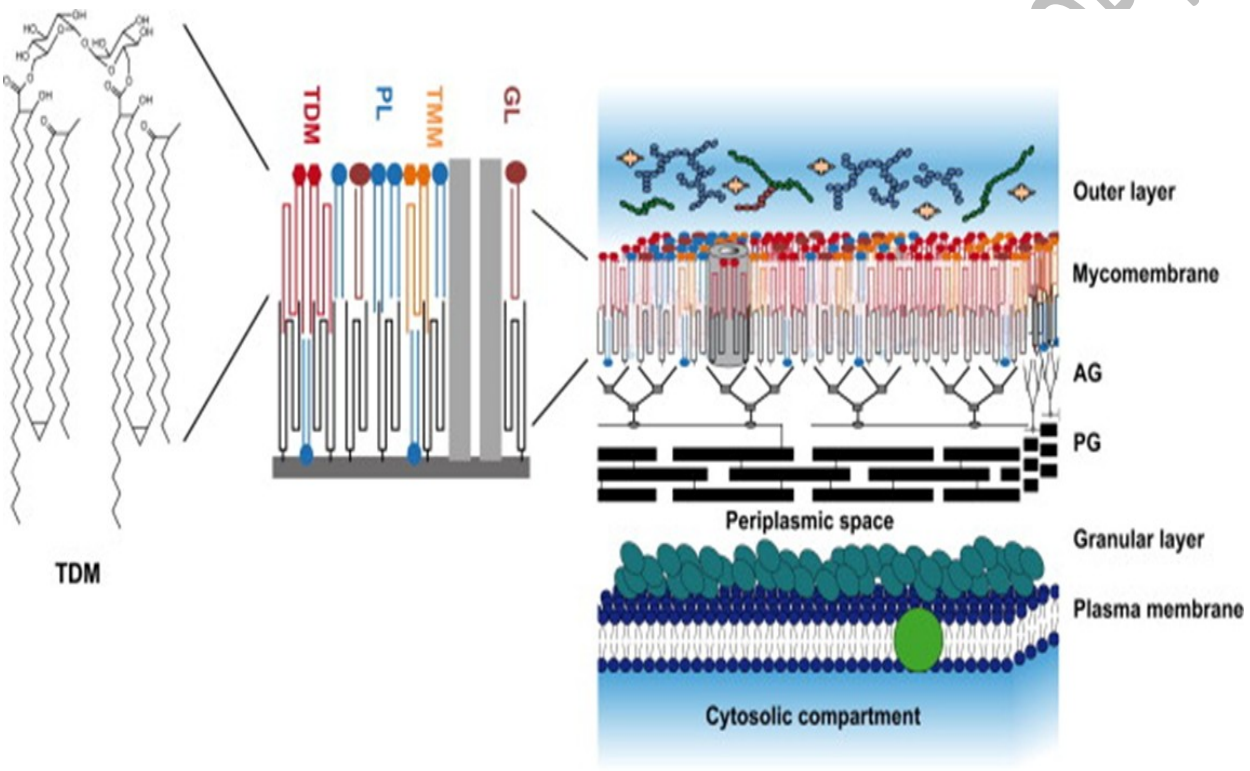


Figure 2
Model of the Mycobacterial Cell Wall (Marrakchi et al. 2014)
The outer layer is mainly composed of glucan and proteins, with only a tiny amount of lipid. The mycomembrane corresponds to the permeability barrier. Its inner leaflet is formed by a parallel arrangement of MA chains (in black) linked to AG that in turn is covalently attached to peptidoglycan (PG); the inner leaflet of the mycomembrane is presumably composed of free lipids that include TDM (in red), TMM (in orange), various glycolipids (GL, in brown), and phospholipid (PL, in blue).

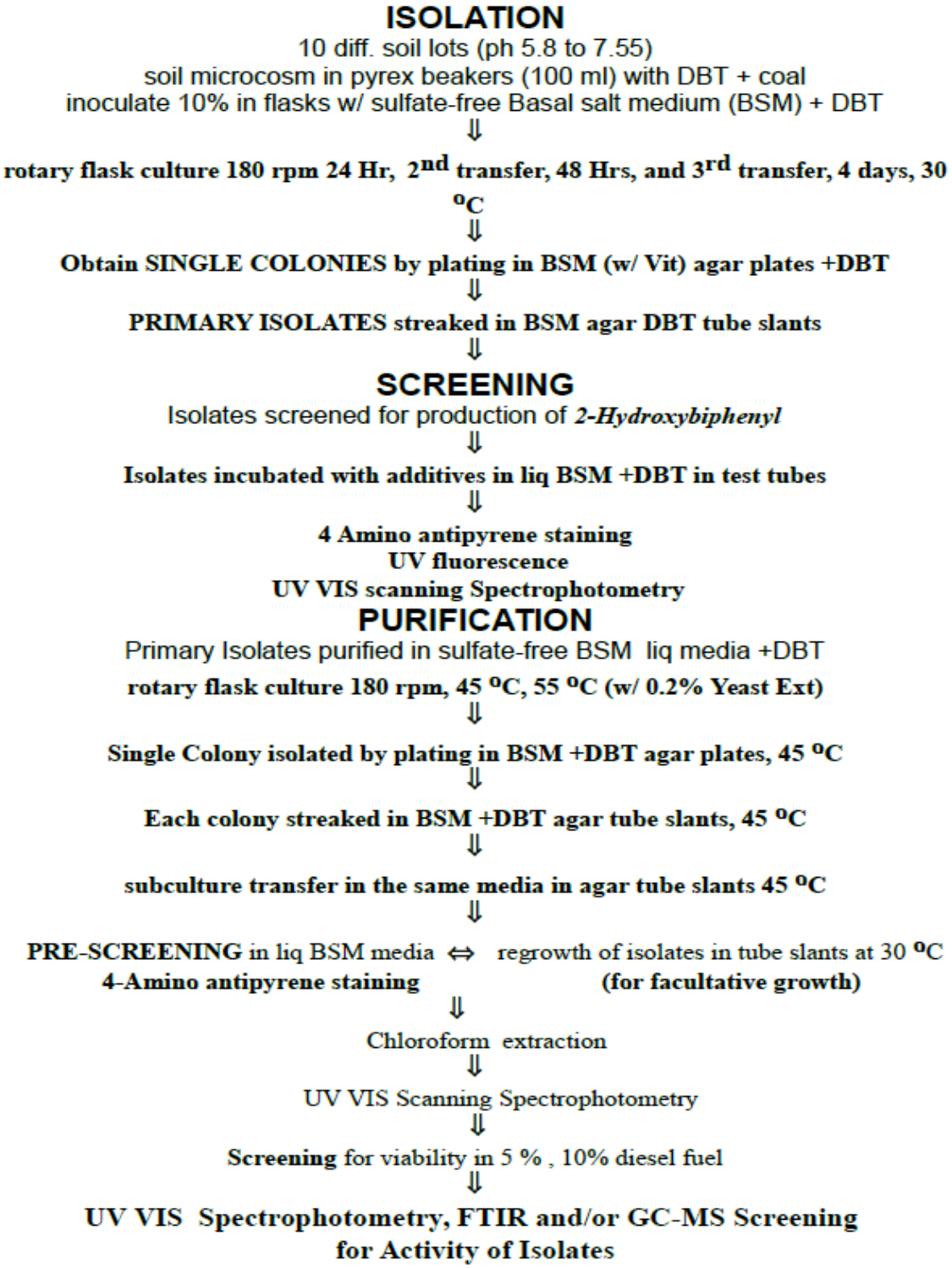


Figure 3. Flow chart of methods.

ISOLATES	G / ETHANOL[b]	G / YEAST EXT[b]	G / MINIMAL M[b]	C.AMINO ACIDS[a] FLUORESCENCE/ 4-AAP
14Mob	++/ +b, +b	++/ - -	++/ --	+b+b/--
142L	++/ --	++/ +b +b	++/ --	+b+b/--
42a	++/ +-b b	++/ +-b b	++/ +- b b	+-b+-b/--
L81a	++/ --	++/ +-b b	++/ --	(N.D.)
14Moa	Slight/ --	++/ +b +b	++/ --	+-b+-b/--
42Moc	++/ --	++/ +-b b	++/ --	(N.D.)
10b1	++/ +b +b	++/ +-b b	++/ --	+b+b/--
14Mob2	++/ +b +b	++/ +b +b	++/ +b +b	+b+b/--
4b	++/ --	++/ +-b b	++/ --	(N.D.)
2b	++/ --	++/ --	++/ --	+-b+-b/--
4µE1	++/ --	-+/ --	++/ --	+-b--/--
4µE2	++/ --	++/ --	++/ --	(N.D.)
2A	Slight/ --	++/ --	Slight/ --	(N.D.)
4A	++/ --	++/ +-b b	++/ --	(N.D.)
5A	Slight/ --	--/ --	--/ --	(N.D.)
3J	Slight/ --	++/ +b +b	Slight/ --	+-b+-b/--
3Jn	++/ +b +b	++/ b b	++/ --	+-b+-b/++*
42e1	++/ +bL +bL	++/ #gL #gL	++/ bL bL	+-b+-b/--
42f1	++/ +bL +bL	++/ #gL, #gL	++/ bL bL	+-b+-b/--
42f2	++/ +bL +bL	++/ #gL, #gL	++/ bL bL	+-b+-b/--
# ISOLATES	8	14	5	13/1

Legend: a = 5ml culture broth (48 hrs), b = agar slant cultures, G = growth(+), b = blue UV fluorescence, L = luminous UV fluorescence, #gL = greenish luminous UV fluorescence, 4- AAP = 4 Aminoantipyrene, * reddish soln

Note: All media contains DBT, Concentration of additives, ethanol and yeast extract 0.2%, casamino acids 0.02% Duration of cultures in ethanol, yeast extract & Min M, 1 day

Table 1. Characteristics of DBT isolates.

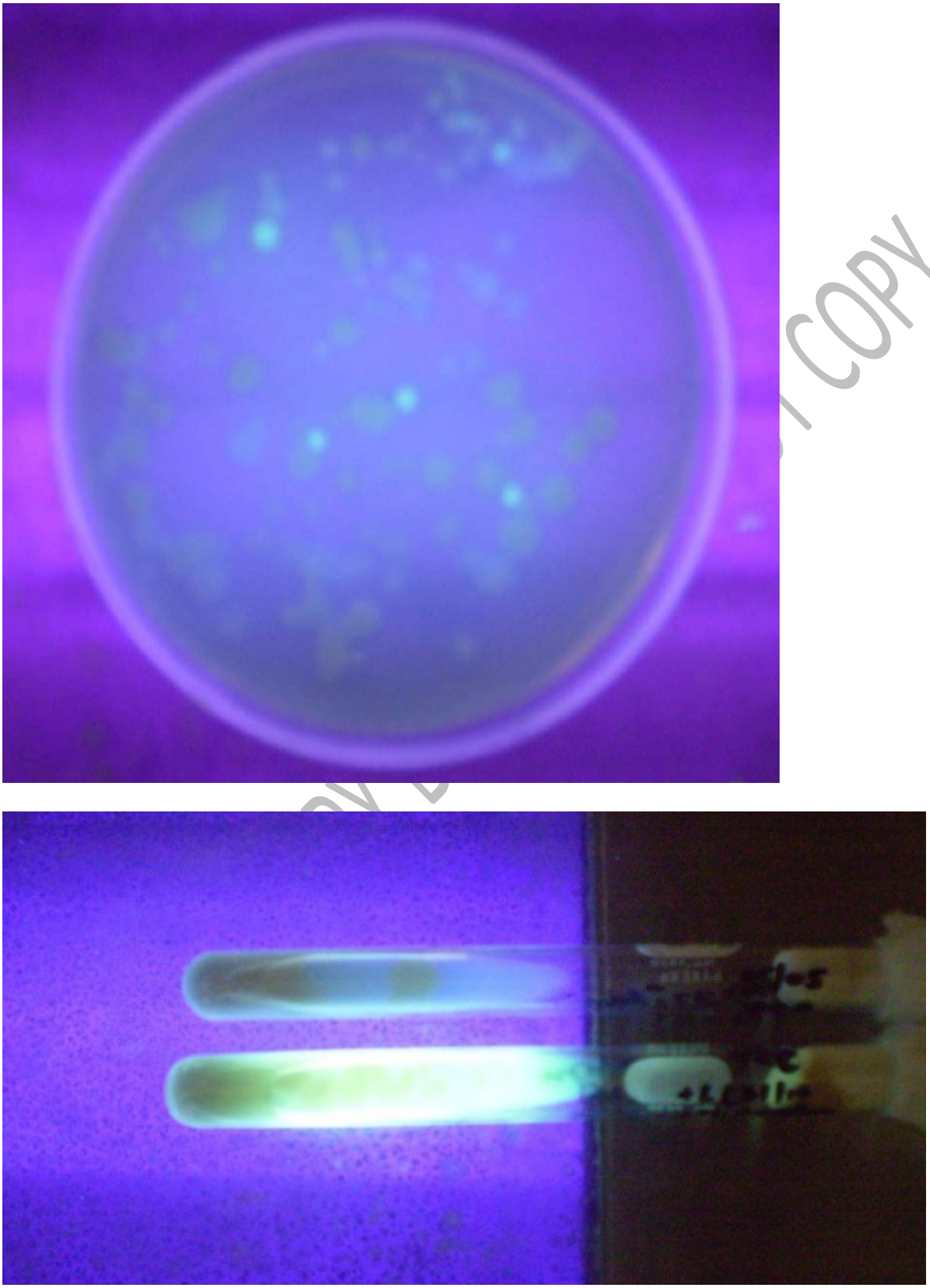


Figure 4. Fluorescent DBT isolates.

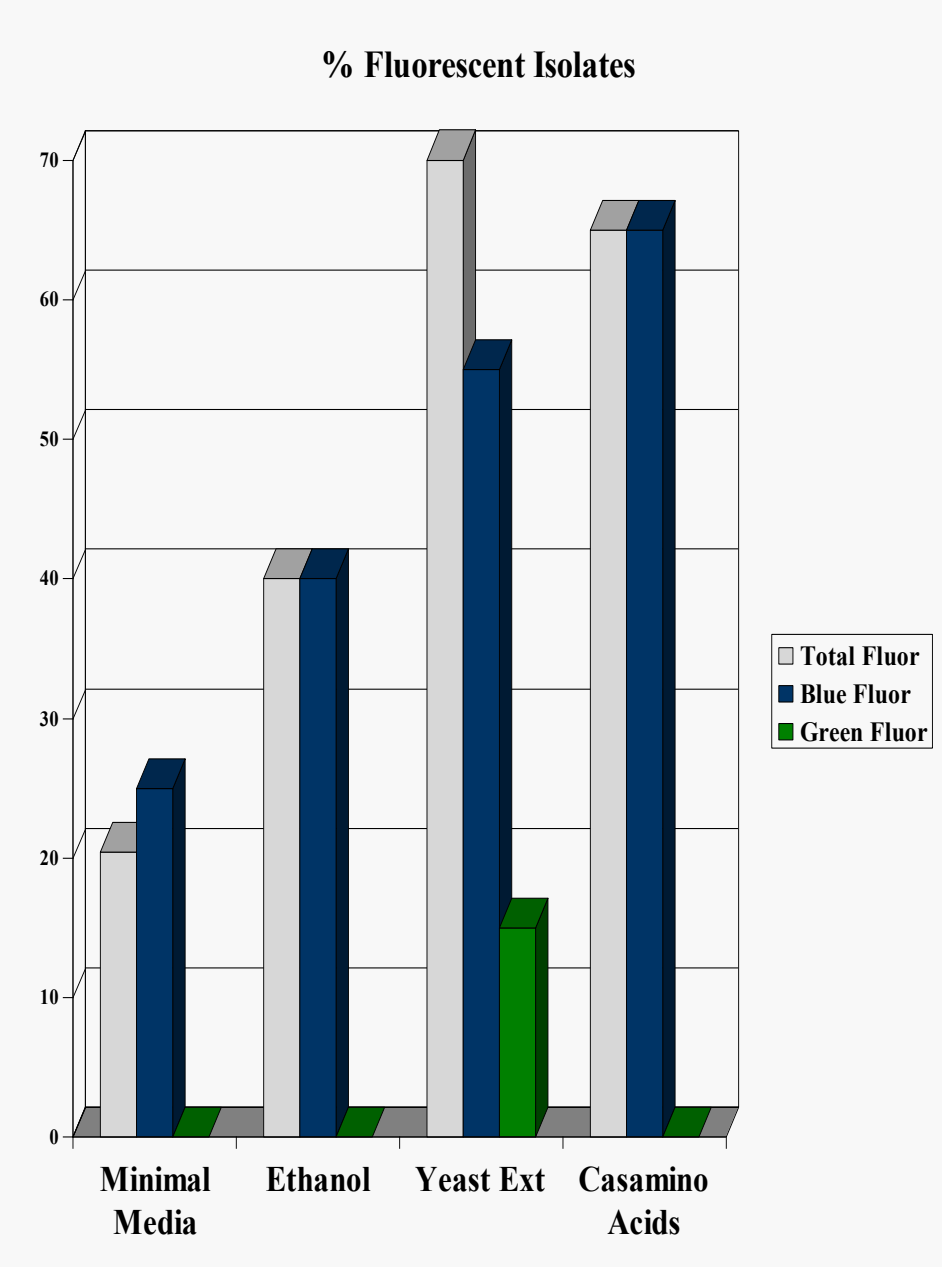


Figure 5. Effect of media components on microbial fluorescence.

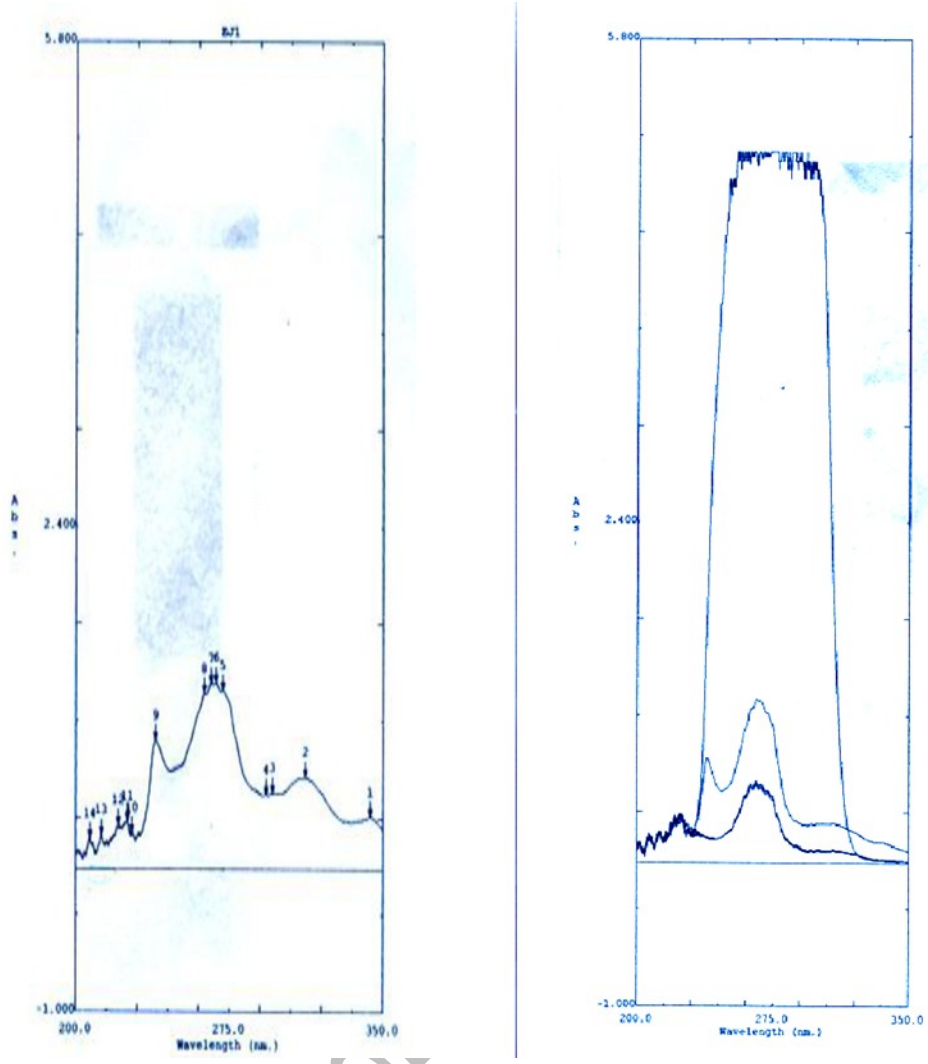


Figure 6 shows the spectral properties of isolate, 4a3 (left) and (right) tracings for 2-Hydroxybiphenyl (2-HBP) upper, with DBT (middle), and control cultures (lower).

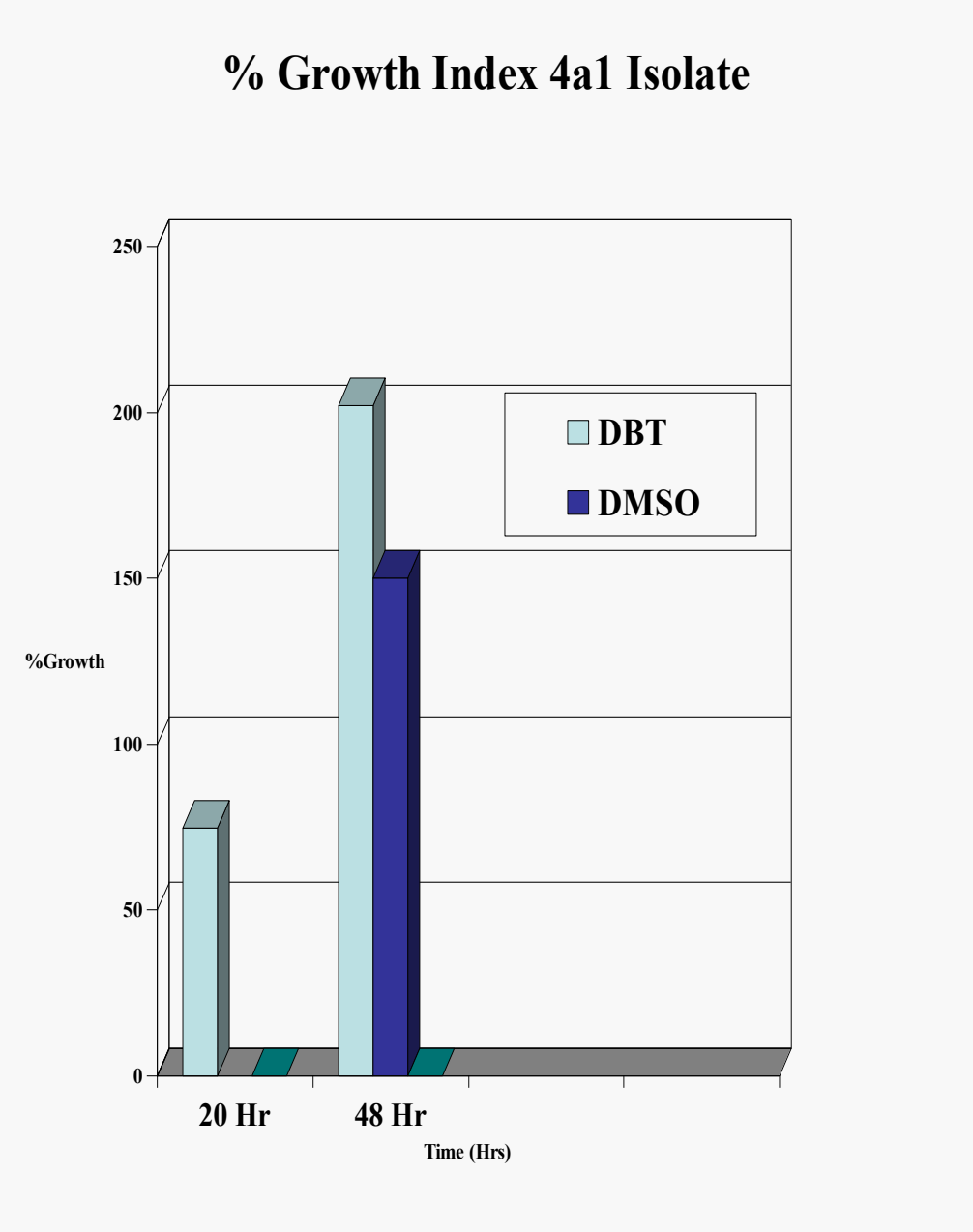


Figure 7. Growth of 4a1 isolate in DBT and DMSO.

COMPARATIVE DIBENZOTHIOPHEN (DBT) ACTIVITIES OF 3Jn1 PRIMARY ISOLATE AT ROOM TEMPERATURE AND AT 45 °C				
SUBSTRATE / ADDITIVES	ABSORBANCE of MAJOR SPECTRAL PEAKS			
	ROOM TEMPERATURE (R.T.)		45 °C	
	262-267 nm	239 nm*	262-267 nm	239 nm*
DBT	0.4753	0.5801	0.1221	0.5656
	0.5109		0.1291	
	0.5063		0.1320	
	0.4620		0.1312	
DBT + YEAST EXTRACT (Y.E.)	0	0.4751	0	0.5376
NOTES:				
Δ239 peak at 45 °C as affected by the addition of Y.E. is 4.95%, which is 3.66x less at R.T.				
Δ239 peak at R.T. cultures as affected by addition of Y.E. is 18.10%				
Y.E. caused disappearance of 262-267nm peak				

Table 2

COMPARATIVE BIODESULFURIZATION ACTIVITIES OF 3Jn3 PRIMARY ISOLATE AT ROOM TEMPERATURE AND AT 45 °C, AFTER PRE-ADAPTATION WITH DIESEL				
MODEL ORGANIC SULFUR SUBSTRATE	ABSORBANCE of MAJOR SPECTRAL PEAKS			
	ROOM TEMPERATURE (R.T.)		45 °C	
	267 nm	239 nm	267 nm	239 nm
DIBENZOTHIOPHENE (DBT)	0.1771	0.6737	0.1188	0.5161
BENZOTHIOPHENE (BT)	0.2883	0.4847	0.1125	0.4400

Table 3

COMPARATIVE ACTIVITIES OF ISOLATES BEFORE AND AFTER PRE-ADAPTATION IN DIESEL				
DIBENZOTHIOPHENE (DBT) ACTIVITIES OF ISOLATES *BEFORE* PRE-ADAPTATION IN DIESEL			DIBENZOTHIOPHENE (DBT) ACTIVITIES OF ISOLATES *AFTER* PRE-ADAPTATION IN DIESEL	
CULTURE ISOLATES	ABSORBANCE of MAJOR SPECTRAL PEAKS		ABSORBANCE of MAJOR SPECTRAL PEAKS	
	267 nm	239 nm	267 nm	239 nm
1 RTa	0.603	0.5044	0.3576	0.4332
1 RTb	0.5735	0.4863	0.1420	0.5051
1 c1	1.0286	0.941	0.2650	0.5098

Table 4

DIBENZOTHIOPHENE (DBT) ACTIVITIES OF ISOLATES AFTER PRE-ADAPTATION IN DIESEL		
CULTURE ISOLATES	ABSORBANCE of MAJOR SPECTRAL PEAKS	
	267 nm	239 nm
1 RTa	0.3576	0.4332
1 RTb	0.1420	0.5051
1 RTc	0.2795	0.4680
1 RTe	0.4042	0.5642
3 b1	0.0862	0.4933
3 a	0.2087	0.4899
4 a3	0.2087	0.4796
3 g	0.1163	0.5071
3 c1	0.1840	0.4697
1 c1	0.2650	0.5098
1 c2	0.01087	0.4410
1 b	0.2204	0.4895
4 a1	0.4191	0.5428
3 b2	0.3672	0.5417
4 a2	0.3002	0.5719
3 e	0.0042	0.5316
4 b1	0.3176	0.6007
3 c2	0.1116	0.6102

Table 5

BENZOTHIOPHENE (BT) ACTIVITIES OF ISOLATES		
CULTURE ISOLATES	ABSORBANCE of MAJOR SPECTRAL PEAKS	
	267 nm	239 nm
1 RTa	0.3216	0.4470
1 RTb	0.2515	0.5368
1 RTc	0.2639	0.4435
1 RTe	0.5054	0.5705
3 b1	0.0351	0.3173
3 a	0.2844	0.5229
4 a3	0.2620	0.4719
3 g	0.1955	0.5053
3 c1	0.1170	0.4475
1 c1	0.0459	0.4950
1 c2	0.1646	0.4215
1 b	0.2723	0.5561
4 a1	0.5254	0.6193
3 b2	0.5269	0.5672
4 a2	0.4909	0.5757
3 e	0.0353	0.4356
4 b1	0.2443	0.5197
3 c2	0.0472	0.5106

Table 6

DIBENZOTHIOPHENE (DBT) ACTIVITIES OF ISOLATES BEFORE PRE-ADAPTATION IN DIESEL

CULTURE ISOLATES	ABSORBANCE of MAJOR SPECTRAL PEAKS (raw data)		
	267 nm		239 nm
	ABS.	nm Range	
1 RTa	1.1708	268, 266	0.7250
1 RTb	1.1057	268, 266, 263	0.7258
1 RTc	1.4090	268, 266	0.9039
1 RTe	1.2005	268, 266	0.7239
3 b1	1.1403	271, 268, 266, 262	0.7617
3 a	NA	NA	NA
4 a3	1.2704	271, 268, 266, 262	0.9004
3 g	1.0647	268, 266	0.7528
3 c1	1.4251	271, 268, 266	0.4697
1 c1	1.0212	271, 268, 266	0.9410
1 c2	1.3896	268, 266	0.9204
1 b	1.3718	268, 266, 262	0.9808
4 a1	1.1798	268, 266	0.7502
3 b2	1.1299	268, 266, 262	0.7396
4 a2	1.1459	268, 266, 262	0.7427
3 e	1.2999	271, 268, 265, 262	1.0082
4 b1	1.1542	268, 266	0.7516
3 c2	1.0846	268, 265, 262	0.7162

Table 7. DBT activities of isolates before pre-adaptation in diesel

DIBENZOTHIOPHENE (DBT) ACTIVITIES OF ISOLATES AFTER PRE-ADAPTATION IN DIESEL				
CULTURE ISOLATES	ABSORBANCE of MAJOR SPECTRAL PEAKS (raw data)			
	267 nm		239 nm	
	ABS.	nm Range	ABS.	nm Range
1 RTa	1.3571	267.00	0.8278	239.70
	1.4134	267.40	0.8899	239.90
	1.0261	266.90	0.8207	239.70
	1.2655		0.8461	
1 RTb	1.2102	267.00	0.7956	239.90
	1.3273	267.00	0.8443	239.80
	1.0677	266.90	0.7764	239.90
	1.2017		0.8054	
1 RTc	1.3202	267.10	0.8170	239.60
	1.2313	267.00	0.8524	239.70
	1.0164	266.90	0.7844	239.70
	1.1893		0.8179	
1 RTe	1.1617	267.10	0.7919	239.60
	1.1537	267.10	0.7872	239.40
	0.9886	267.10	0.7906	239.40
	1.1013		0.7899	
3 b1	1.3466	267.00	0.8958	239.70
	1.1955	267.10	0.8197	239.60
	1.1886	267.10	0.8220	239.70
	1.2436		0.8458	
3 a	1.0120	267.10	0.7119	239.50
	1.2512	267.10	0.8389	239.60
	1.0823	267.10	0.7820	239.40
	1.1152		0.7776	
4 a3	1.2728	267.10	0.7711	239.80
	1.3292	267.10	0.8251	239.90
	1.1987	267.00	0.8048	239.80
	1.2669		0.8003	
3 g	1.2176	267.10	0.8036	239.50
	1.2865	267.10	0.8113	239.50
	1.0312	267.10	0.7701	239.50
	1.1784		0.7950	
3 c1	1.1871	267.00	0.8624	239.60
	1.3556	267.10	0.8883	239.60
	1.2549	267.00	0.8247	239.50
	1.2659		0.8585	

Table 8. DBT activities of isolates after pre-adaptation in diesel

DIBENZOTHIOPHENE (DBT) ACTIVITIES OF ISOLATES AFTER PRE-ADAPTATION IN DIESEL

CULTURE ISOLATES	ABSORBANCE of MAJOR SPECTRAL PEAKS (raw data)			
	267 nm		239 nm	
	ABS.	nm Range	ABS.	nm Range
1 c1	1.3295 1.3415 1.2367 1.3026	267.00 267.00 266.90	0.8354 0.8824 0.9106 0.8761	239.90 239.90 240.00
1 c2	1.2346 1.1601 1.1668 1.1872	266.90 267.00 266.90	0.8268 0.8145 0.8266 0.8226	239.80 239.80 239.70
1 b	1.1701 1.1473 1.2549 1.1908	267.00 266.90 267.00	0.7901 0.8082 0.8153 0.8045	239.60 239.70 239.60
4 a1	1.1573 1.1469 1.3351 1.2131	267.00 267.00 267.00	0.7745 0.7984 0.8314 0.8014	239.60 239.60 239.60
3 b2	1.0376 1.1658 1.3287 1.1774	267.00 267.00 267.00	0.7592 0.8263 0.8650 0.8168	239.50 239.60 239.70
4 a2	1.0145 1.1704 1.2561 1.1470	266.90 266.90 266.90	0.7692 0.8495 0.8766 0.8318	239.50 239.60 239.60
3 e	1.0235 1.2535 1.2043 1.1604	266.90 266.90 267.00	0.8515 0.9303 0.9440 0.9086	239.70 239.70 239.60
4 b1	1.2586 1.3038 1.3167 1.2930	267.00 267.00 267.00	0.9124 0.9377 0.9670 0.9390	239.60 239.70 239.60
3 c2	1.1332 1.3077 1.4079 1.2829	266.90 266.90 266.90	0.9663 1.0446 1.0214 1.0108	239.60 239.70 239.80

Table 9. DBT activities of isolates after pre-adaptation in diesel

PEAK (DBT) ABSORBANCE ACTIVITIES OF MAJOR SPECTRAL PEAK BEFORE AND AFTER PRE-ADAPTATION IN DIESEL		
CULTURE ISOLATES	**BEFORE** PRE-ADAPTATION [*271, 267-268, 262*] nm range	**AFTER** PRE-ADAPTATION [*266-267*] nm range
	nm Recorded	**nm Recorded**
1 RTa	268 266	267.00 267.40 266.90
1 RTb	268 266 263	267.00 267.00 266.90
1 RTc	268 266	267.10 267.00 266.90
1 RTe	268 266	267.10 267.10 267.10
3 b1	271 268 266 262	267.00 267.10 267.10
3 a	NA	267.10 267.10 267.10
4 a3	271 268 266 262	267.10 267.10 267.00
3 g	268 266	267.10 267.10 267.10
3 c1	271 268 266	267.00 267.10 267.00
1 c1	271 268 266	267.00 267.00 266.90
1 c2	268 266	266.90 267.00 266.90
1 b	268 266 262	267.00 266.90 267.00
4 a1	268 266	267.00 267.00 267.00
3 b2	268 266 262	267.00 267.00 267.00
4 a2	268 266 262	266.90 266.90 266.90
3 e	271 268 265 262	266.90 266.90 267.00
4 b1	268 266	267.00 267.00 267.00
3 c2	268 265 262	266.90 266.90 266.90

Table 10. Peak DBT absorbance activities of major spectral band before and after pre-adaptation in diesel

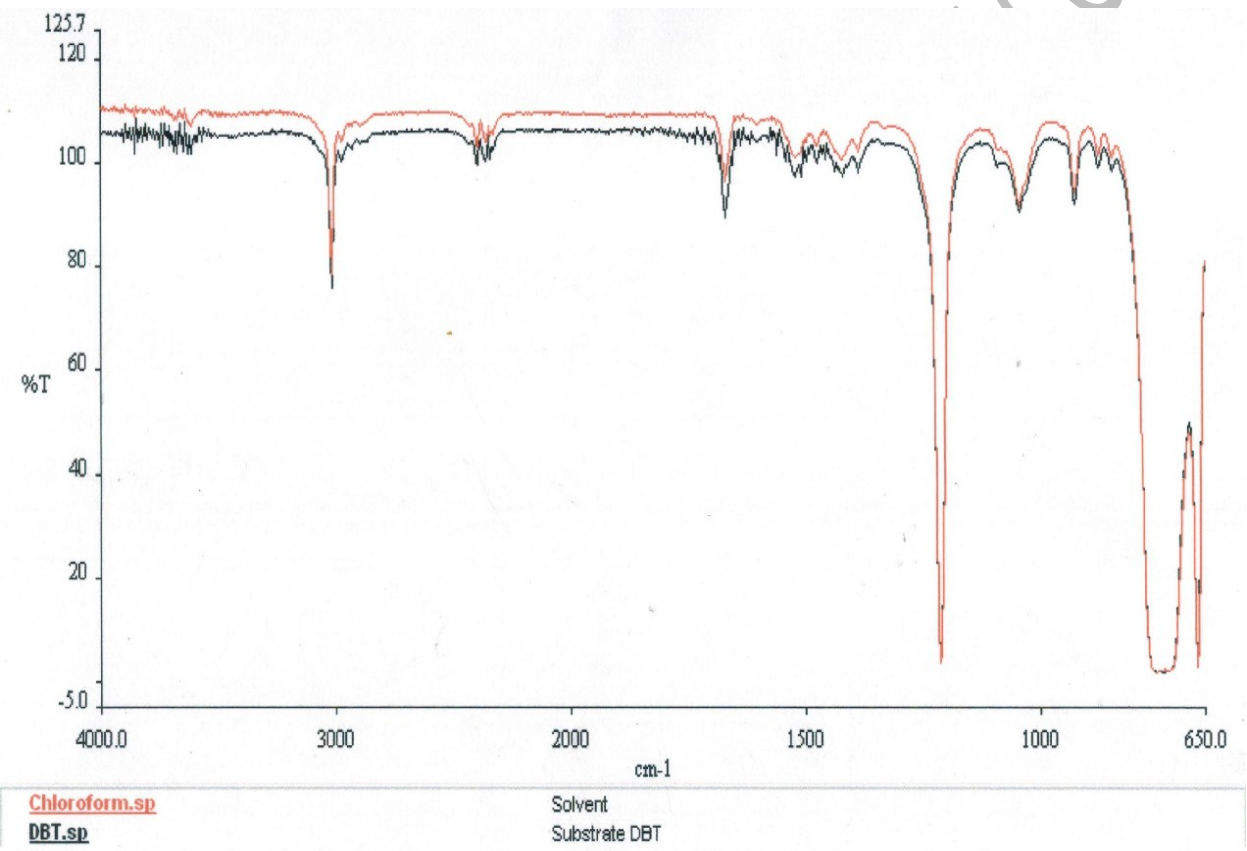


Figure 8. Effect of solvent on FTIR spectra.

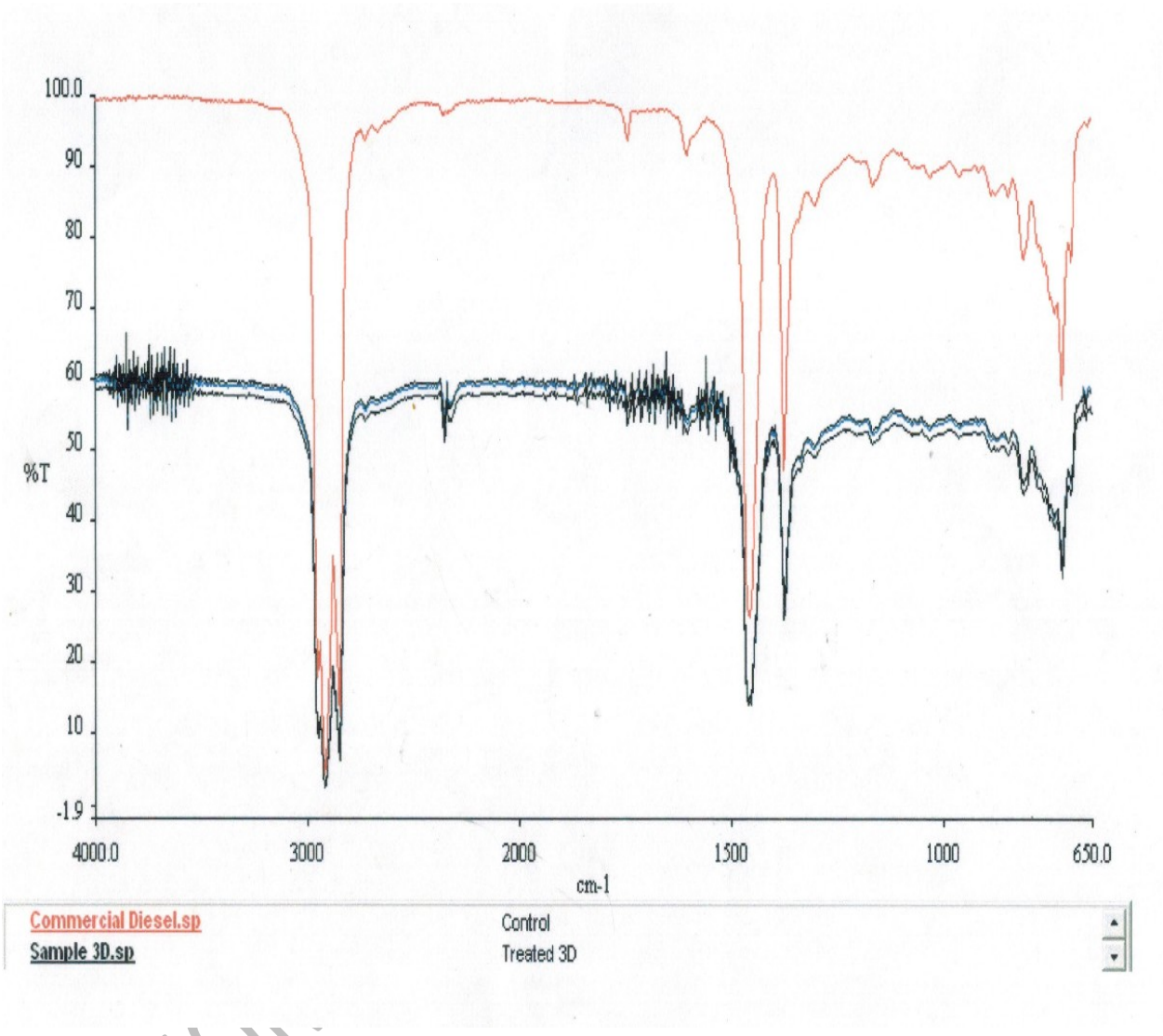


Figure 9. FTIR ATR Spectra of diesel oil (upper trace in light red) and microbial treated diesel (lower trace) show that major resolvable peaks are conserved during microbial processing without affecting the calorific value of the fuel.

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Operator: itdi
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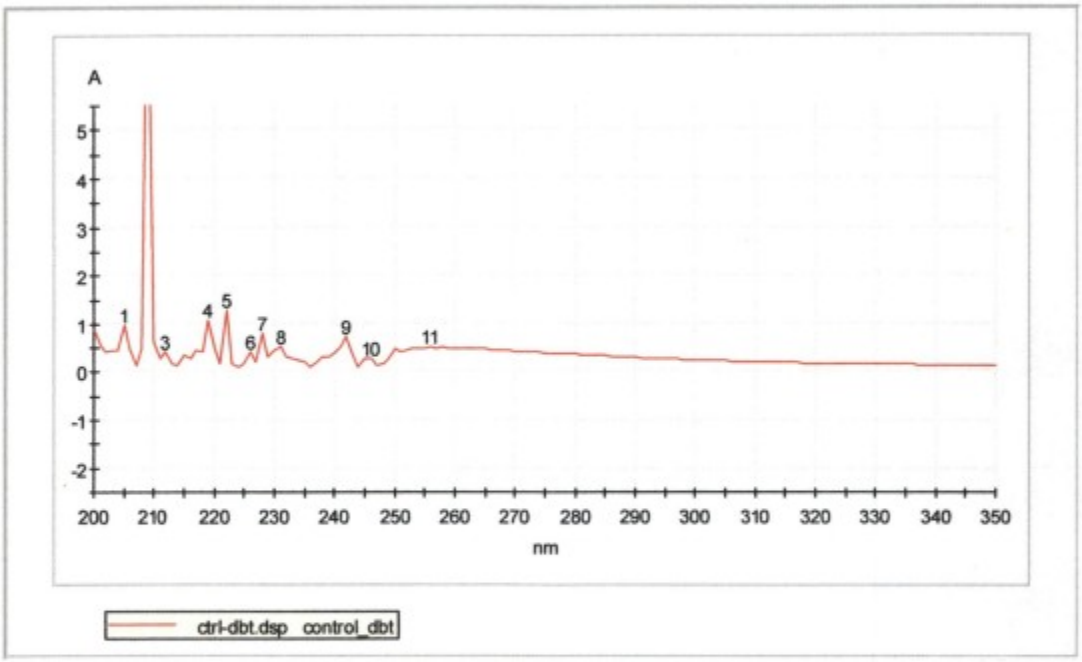
Spectrophotometer: Evolution 60
Serial number: 2Q4K337001
Firmware: 1.007



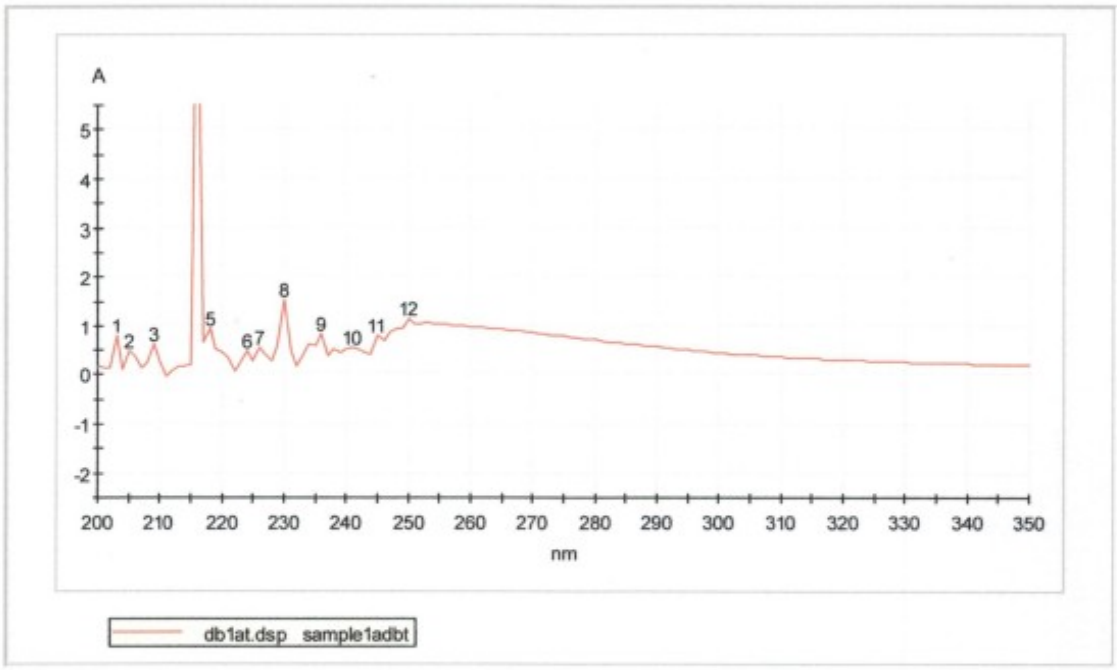
ctrl-dbt.dsp control_dbt

Maxima Threshold: 0.1 A

1	205 nm;	0.954 A	2	209 nm;	10.000 A	3	212 nm;	0.383 A
4	219 nm;	1.048 A	5	222 nm;	1.288 A	6	226 nm;	0.404 A
7	228 nm;	0.787 A	8	231 nm;	0.505 A	9	242 nm;	0.721 A
10	246 nm;	0.262 A	11	256 nm;	0.492 A			

Figure 10. Control with DBT (UV scanning using Evolution 60, Thermo Scientific)

Spectrum: db1at.dsp
Description: sample1adbt
Operator: itdi
Created: 2/10/2010 3:54:56 PM
Spectrophotometer: Evolution 60
Serial number: 2Q4K337001
Firmware: 1.007



db1at.dsp sampleladbt

Maxima	Threshold: 0.1 A						
1	203 nm;	0.794 A	2	205 nm;	0.479 A	3	209 nm;	0.602 A
4	216 nm;	10.000 A	5	218 nm;	0.941 A	6	224 nm;	0.459 A
7	226 nm;	0.543 A	8	230 nm;	1.518 A	9	236 nm;	0.823 A
10	241 nm;	0.523 A	11	245 nm;	0.797 A	12	250 nm;	1.132 A

Figure 11. Microbial treatment of DBT (0.5 mM) resulted in the disappearance of the DBT peak (209 nm), which indicated a direct microbial conversion of DBT, resulting a product (2-HBP) with a major peak at 216 nm. (Samples in flasks with three replicates were incubated at rpm 180 for 24 h, at 45 ^{o}C. Cell-free cultures were extracted with ethyl acetate and chloroform added with equal volume, thereafter scanned with UV Spectrophotometry using Evolution 60, Thermo Scientific).

STRAINS	267 nm		239 nm	
	DBT	BT	DBT	BT
1RTa				
1RTb	↓	↑		
1RTc				
1RTe	↓	↑		
3b1	↑	↓	↑	↓
3 a				
4 a3				
3 g				
3 c1				
1 c1	↑	↓		
1 c2	↓	↑		
1 b			↓	↑
4 a1	↓	↑	↓	↑
3 b2	↓	↑		
4 a2	↓	↑		
3 e	↓↓	↑	↑	↓
4 b1	↑	↓	↑	↓
3 c2	↑	↓	↑	↓
% EXPRESSION	↑ (4/18) 22.% ↓ (7/18) 39.%	↑ (7/18) 39.% ↓ (4/18) 22.%		↑ (2/18) 11.1% ↓ (4/18) 22.%

Figure 12. Expression profile of isolated strains
Data shaded in green showed no significant difference (<20%)

	267 nm		239 nm	
	DBT/ Spent Media	Crude/ Bunker Fuel/ Spent Media	DBT/ Spent Media	Crude/ Bunker Fuel/ Spent Media
% Biodesulfurization Activity	173.10%	132.25%	175.23%	211.36%

Figure 13. Relative Biodesulfurization Activity

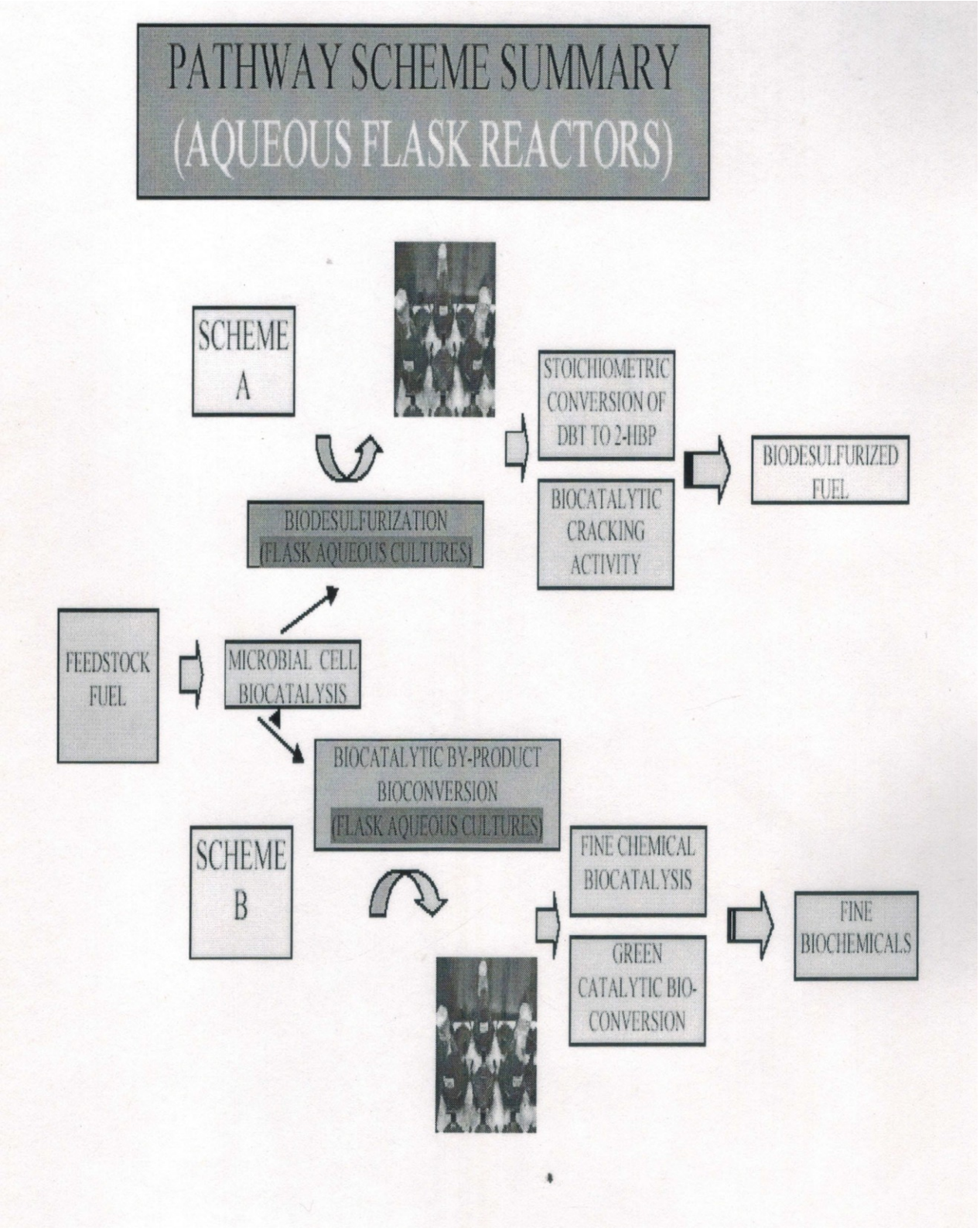

PATHWAY SCHEME SUMMARY
(AQUEOUS FLASK REACTORS)
SCHEME A
STOICHIOMETRIC CONVERSION OF DBT TO 2-HBP
BIODESULFURIZED FUEL
BIOCATALYTIC CRACKING ACTIVITY
BIODESULFURIZATION (FLASK AQUEOUS CULTURES)
FEEDSTOCK FUEL
MICROBIAL CELL BIOCATALYSIS
BIOCATALYTIC BY-PRODUCT BIOCONVERSION (FLASK AQUEOUS CULTURES)
SCHEME B
FINE CHEMICAL BIOCATALYSIS
FINE BIOCHEMICALS
GREEN CATALYTIC BIO-CONVERSION

Figure 14. Pathway Scheme Summary (Aqueous flask reactors)

CHAPTER 2

EFFLUENT BIOGAS PRODUCTION FROM SWINE MANURE IN TWO-STAGE PROCESS

NOEL M. UNCIANO, FLORENCIA CUBOL, & DAVID HERRERA

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ABSTRACT

A two-stage bioreactor system consisting of two 30-L plastic drum digesters connected in series was setup and batch-fed with organic load of: 17% organic swine manure, 30% fresh inoculum, and 53% water. The hydraulic retention time (HRT) for digester 1 was 6-8 days; digester 2 was 13-16 days. Using principal component analysis (PCA), we showed that the top 5 bioprocess parameters were total solids (TS), total suspended solids (TSS), chemical oxygen demand (COD), biochemical oxygen demand (BOD), and volume of gas. Significant positive correlations were observed with TS, TSS, COD, and BOD; volume of gas was negatively correlated and increased twice compared to digester 1. Three of the parameters namely: TS, TSS, BOD exhibited significant % reduction. Data clustering was also observed. The bioprocess efficiency for the two-stage reactors was computed to be 73.89%. The average gas volumetric production rates were 15.875 L/ L Reactor1 or 30.45 L/ L Reactor2. Based on the cumulative gas production, the theoretical Biochemical Methane Potential (BMP) on the average was 880.2289 x 10^3 ml CH_4/g COD, which represented only about half of the methane value.

SIGNIFICANCE:

In the Philippines, an estimated national amount of swine manure production of about 44,478 tons per day or 16.23 M tons per year would require sustainable waste disposal technology. The two-stage digestion is a process configuration using separate reactors,

one for liquefaction (hydrolysis) and acidification and the other for biomethanation. The primary source of microbial inoculum will be from manure slurry. The concept of two-phase digestion (Pohland and Ghosh 1971) also known as acid phase digestion involves a first phase that is usually operated at a short hydraulic retention time with a lower pH while methane formation stage is operated at a long hydraulic retention times (10-30 days) at a near neutral pH to maintain favorable conditions for methanogenic bacterial. Process stabilization will be maintained by various bio-physicochemical parameters.

The waste management of manure will mitigate greenhouse gasses (GHG) from livestock and could increase the profitability of production systems using enhanced methane production and thus will provide a cost effective alternative renewable energy source for clean fuel.

Although considered in the niche market (Teune B, Orprecio J et al. 2010), together with landfill gasification and banana-waste to biomethane, it is considered to be the cleanest, more efficient and most-climate neutral transport fuel in the market (EU well-to-wheel study, 2011).

The relatively high cost of methane digesters fabrication is still a barrier and a challenge to find and use low cost materials and process for this conversion remains an option to be considered in the application of micro-bioprocessing in renewable clean fuel production and waste management.

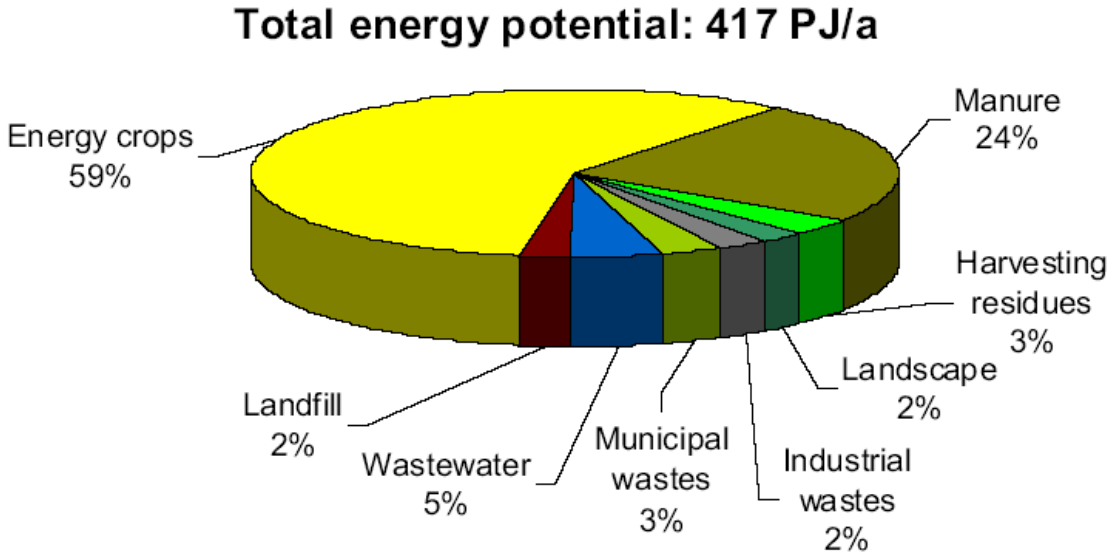
- *OBJECTIVES:*

  To develop a bench-scale 20-liter biogas production using a two-stage process and determine the operational process parameters.

- *LITERATURE*

Currently, the majority of the world energy needs are supplied through carbon-containing fossil fuel sources such as coal, natural gas, and oil. Due to the economic conditions escalating oil prices and negative environmental impacts, government initiatives in many countries are focusing on the increased use of various renewable energies including solar, wind, biomass, hydro-power, tidal energy, and energy from waste.

Over 1.8 billion tonnes of waste including households, industry and agriculture, etc. are generated each year in Europe alone. Biogas production from waste provides an environmentally friendly way for waste management as well as production of sustainable renewable energy.



(Usable biogas potential in Germany, FNR 2008; Cited in Weiland 2010)

Anaerobic digestion (AD) using manure for biogas production is one of the most promising uses of biomass wastes because it provides a source of energy while simultaneously resolving ecological and agrochemical issues. Depending on the feedstock, the biogas produced from anaerobic digestion of waste usually contains 40-70% methane (CH_4) and 30-50% carbon dioxide (CO_2).

The basic reasons to process manure to biogas using anaeroabic digestion (AD) are as follows: The first reason is to recover useable energy that contributes no net carbon to the atmosphere. A second reason to use AD for biogas is to reduce the risk from pathogens. A third and prospective benefit from AD processing of manure is the potential to recover nutrients from digestate, leaving a disposable water stream.

Studies of Møller et al., (2004) found that at 15°C, aerobic processes in the surface layers of the stored manure slurry are more dominant than anaerobic processes, while at a higher temperature (20°C), the aerobic and anaerobic processes are of almost equal importance. In general, the anaerobic processes are more dominant during storage of cattle manure than during storage of pig manure.

Small scale anaerobic digesters provide potential wealth of benefits and solution to: a) poor indoor air quality and subsequent chronic health problems, b) unequal exposure to hazards by gender, c) the need for a cooking fuel, d) deforestation for fuel use, e) lack of treatment of animal waste, f) expensive inorganic fertilizers, g) mitigation of methane released into the atmosphere, and h) reduced amount of residuals for disposal, compared with aerobic treatment. However, many small scale anaerobic digesters in developing countries fail for various reasons, including: design, high capital cost, construction, maintenance, user needs, operational problems, or availability of materials for maintenance.

The anaerobic process begins with a group of fermentative bacteria that excrete enzymes that break down macromolecules in the reactor. Next, a different group of fermentative bacteria partially oxidizes the organic acids produced during fermentation

into volatile fatty acids (with less than two carbons) in a process called acidogenesis . The volatile fatty acids of significance formed in this step are: propionic acid, n-butyric acid, and isobutyric acid. Alcohol formation also takes place during this step.

Hydrogen- producing acetogenic bacteria convert the volatile fatty acids and ethanol produced in acidogenesis into acetic acid, hydrogen, and carbon dioxide in a process called acetogenesis. In order for acetogenesis to be a thermodynamically favorable process and for the reaction to proceed in a forward direction, the partial pressure of hydrogen in the system must be less than 10^{-3} atm. Hydrogen is scavenged by methanogenic archaea which, in turn, results in a low partial pressure of hydrogen and maintains a thermodynamically favorable acetogenesis process.

Methane (CH_4) production, which is an H_2-consuming reaction requires a reduction in the level of H_2 partial pressure to drive the reaction forward. In anaerobic digestion the bioconversion of fatty acids, such as propionate stops when the H_2 concentration is above 10^{-4} atm; higher H_2 concentrations can result in increased concentrations of propionic acid and other carboxylic acids in the digester. Increased acid concentration can cause the pH to decrease and inhibit or stop CH_4 production.

Methane fermentation is a complex process, which can be divided up into four phases: hydrolysis, acidogenesis, acetogenesis/dehydrogenation, and methanation. The individual degradation steps are carried out by different consortia of microorganisms requirements on the environment. A complex consortium of microorganisms participates in the hydrolysis and fermentation of organic material. Most of the bacteria are strict anaerobes such as *Bacteriocides, Clostridia, and Bifidobacteria*. Furthermore, some facultative anaerobes such as Streptococci and Enterobacteriaceae take part. The higher volatile fatty acids are converted into acetate and hydrogen by obligate hydrogen-producing acetogenic bacteria. The hydrogen-producing acetogenic bacteria are not well characterized. Typical homoacetogenic bacteria are *Acetobacterium woodii* and *Clostridium aceticum.* The accumulation of hydrogen can inhibit the

metabolism of the acetogenic bacteria. The maintenance of an extremely low partial pressure of hydrogen is, therefore, essential for the acetogenic and H_2-producing bacteria. Although many microbial details of metabolic networks in a methanogenic consortium are not clear, present knowledge suggests that hydrogen may be a limiting substrate for methanogens. This assumption is based on the fact that addition of H_2-producing bacteria to the natural biogas-producing consortium increases the daily biogas production. At the end of the degradation chain, two groups of methanogenic bacteria produce methane from acetate or hydrogen and carbon dioxide. These bacteria are strict anaerobes and require a lower redox potential for growth than most other anaerobic bacteria. Only few species are able to degrade acetate into CH_4 and CO_2, e.g., *Methanosarcina barkeri*, *Metanonococcus mazei*, and *Methanotrix soehngenii*, whereas all methanogenic bacteria are able to use hydrogen to form methane.

Much is known about the basic metabolism in different types of anaerobic digestion processes, but little is known about the microbes responsible for these processes. Only few percent of bacteria and archaea have so far been isolated, but little is known about the dynamics and interactions between these microorganisms. The lack of knowledge results sometimes in malfunctions and unexplainable failures of biogas fermenters. With molecular techniques, more information can be received about the community structures in anaerobic processes. A recent report detected 68 taxonomic groups of methanogens in samples from ten agricultural biogas plants. This showed that hydrogenotrophic methanogens dominate in most agricultural biogas plants. A high share of acetogenic methanogens can be found obviously only in biogas plants which are operated at low ammonia concentrations.

Research literature showed that methanogenic diversity is lower in plants operating at thermophilic temperatures. Although the growth rate of methanogenic bacteria is higher at thermophilic process temperatures, these processes are more sensitive to temperature fluctuations and require longer time to adapt to a new temperature. Furthermore, these result in a larger degree of imbalance and a higher risk for ammonia

inhibition. Mesophilic bacteria tolerate temperature fluctuations of +/-3 °C without significant reductions in methane production. An option for thermophilic digester is to load to a higher degree or operate at a lower hydraulic retention time (HRT).

Methane formation takes place within a relatively narrow pH interval, from about 6.5 to 8.5 with an optimum interval between 7.0 and 8.0. The process is severely inhibited if the pH decreases below 6.0 or rises above 8.5.

Acetic acid is usually present in higher concentration than other fatty acids, but propionic and butyric acids are more inhibitory to methanogens. This inhibition is associated with the un-dissociated form. Thus, the inhibiting effect of VFAs is much higher in systems of low pH value.

Methane can be generated via two different pathways during methanogenesis. One pathway takes the substrates hydrogen and carbon dioxide and forms methane through hydrogenetrophic methanogenesis. Some of the hydrogen and carbon dioxide is converted into acetate through homoacetogenesis. The remaining pathway converts acetate into methane and carbon dioxide in a process called acetotrophic methanogenesis.

Why do we study the two-stage anaerobic digestion process? Recent reports showed that methane yields and production were shown to be higher in the two-stage reactors than in the one-stage reactor, however results of the continuous experiments showed lower yields than those obtained in batch conditions (Bertin et al. 2013) and as suggested (Llansing et al. 2010) seasonal producers could utilize low-cost digesters operating at a lower temperature range of 24 to 26oC and sustained at 50–78% of normal methane production levels for 75 day at a co-digestion.

This concept of two-phase digestion is also known as acid phase digestion. The first phase is usually operated at a short SRT (4-12hr) and at lower pH (≤6), while methane

formation stage is operated at long SRTs (10-30 days) and at neutral pH to maintain favorable environment for methanogenic bacteria.

Pohland and Ghosh (1971) first proposed the physical separation of the process into an acidogenic and a methanogenic stage. This provides enhanced stability to the different groups of microorganisms for better process control, and to extract more net energy from the system. In a single-stage anaerobic digestion process, a variety of higher organic acids, such as propionic, butyric, and lactic, as well as alcohols and ketones, are formed during the breakdown of the organic substrates by acidogens. However, in a well operated process, these products are mostly converted to acetic acid and hydrogen, which, in turn, are converted to methane gas. On the other hand, in a two-stage anaerobic digestion process, the end products from acidification stage are usually ideal for anaerobic treatment with high VFAs concentrations. It was suggested that HRT of 3 days is needed for activated sludge achieving the optimum acidogenic fermentation in the two-phase anaerobic digestion.

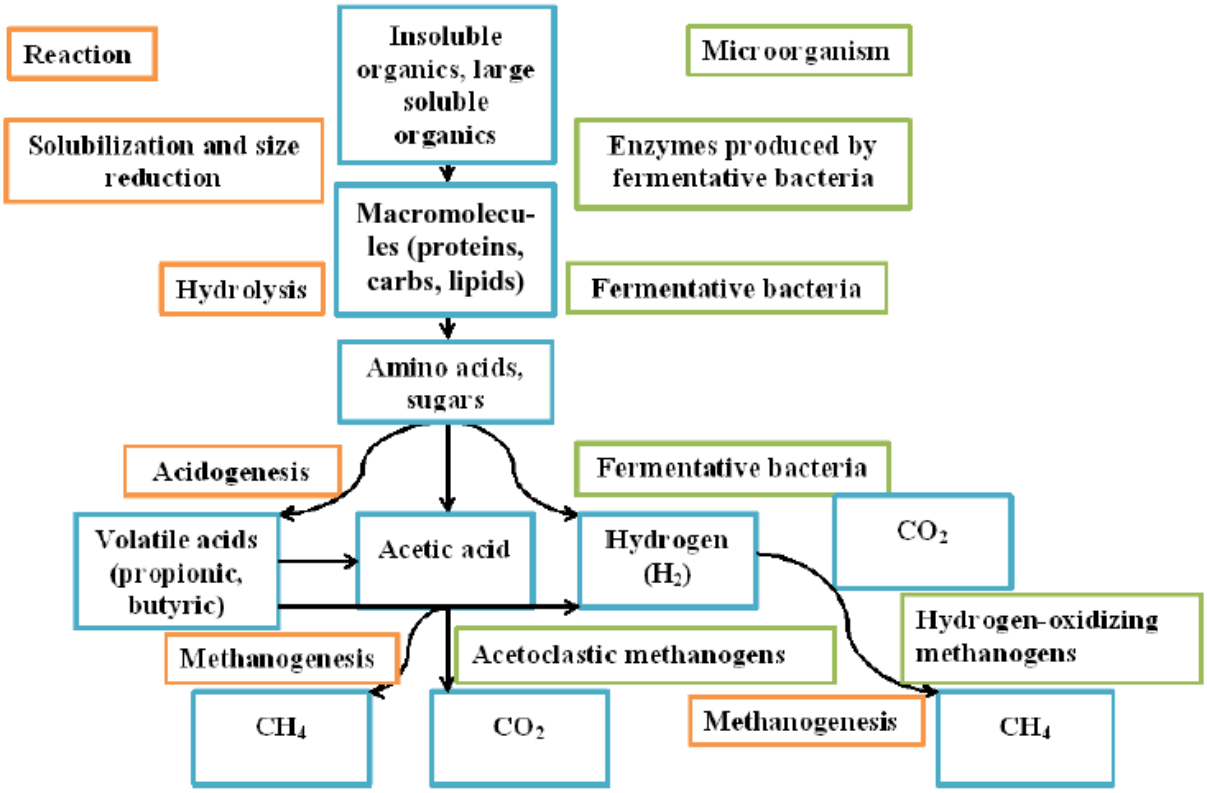
It is known that in a methane reactor, 67% of the methane is produced by acetate-utilizing methanogens and 33% is produced by hydrogenophilic methanogens. ethanol that are not as favourable as acetate for methane production. Various authors achieved more than 10% increase in methane production using a two-stage process compared to a single-stage. Despite their higher loading rates, improved process stability and flexibility, there are relatively few commercial two-stage anaerobic digestion units.

There are some disadvantages to the anaerobic digestion process. First, small scale anaerobic digestion requires the addition of water, which is limiting especially during the dry season. Anaerobic digestion takes more time to start- up the process because methanogens have slower growth kinetics. High effluent BOD_5 concentrations prevent direct discharge into water bodies. Anaerobic digestion may require the addition of alkalinity (in the form of sodium bicarbonate) to reach levels of 2000- 3000 mg/L as $CaCO_3$ in order to maintain an optimal pH. Reaction rates in the anaerobic digestion processes are much more sensitive to changes in temperature. For this reason, a

stable operating temperature is very important, and changes in temperature of less than 0.5 ^{o}C/day are recommended. Higher capital costs are associated with anaerobic digestion than with aerobic treatment because a larger reactor volume is required for anaerobic treatment and because of the additional infrastructure required for methane capture and energy use. Anaerobic digestion is much more vulnerable to upsets from toxic compounds found in the waste stream and there is a potential for the production of corrosive gases and odors. Also, the theoretical higher biogas yields are limited since the acidogenic phase separation prevents the hydrogen to methane pathway.

For anaerobic digestion to be cost-competitive, the minimum waste chemical oxygen demand (COD) should be above 1500 to 2000 mg/L. However, the process performance such as methane yield, solids reduction efficiency primarily depends on the level of biodegradable organics in the waste, or biochemical oxygen demand (BOD). The most common anaerobic bioreactor designs that provide biomass retention (thereby preventing failure of digester) are the upflow anaerobic sludge bed (UASB), expanded granular sludge bed (EGSB), fluidized bed bioreactor (FBR), and anaerobic membrane bioreactor (AnMBR).

A general scheme for anaerobic digestion process is shown below.



Anaerobic Digestion Process Diagram. (after Grady et al., 2009).

Gas storage must be available for the liquid volume change in anaerobic digestion to prevent exposure to flammable volatiles and avoid accidents. It is recommended that the volume in the reactor for gas storage space should be 1/5 the volume of the solid and liquid volume in the reactor. Biogas storage containers should be durable and resistant to corrosion. If methane gas is released uncontrolled, methane and air can form an explosive mixture that can spontaneously combust at high temperatures (Tchobanoglous et al., 2003).

Methane yield can be expressed in several ways. The term "methane productivity" is used to indicate the yield of methane per unit of a variable term such as volatile solids (VS) destroyed, VS loaded, volume or animal production. Methane productivity measured in terms of VS destroyed corresponds to the theoretical methane yield (Bu) by a complete degradation of all organics in the manure. The theoretical methane

potential can be calculated from Bushwell's formula (Symons and Bushwell, 1933). Methane productivity in terms of VS loaded is referred to as the ultimate methane yield (Bo). The ultimate methane yield will always be lower than the theoretical yield due to the fact that because a fraction of the substrate is used to synthesize bacterial mass, a fraction of the organic material will be lost in the effluent, and lignin-containing compounds are only degraded to a limited degree. Inhibition of the biological process by inhibitors such as ammonia and VFA is another factor which means that the methane yield is reduced, compared with the yield which would be produced if inhibition was not present. It has been observed that both the ultimate methane yield (Bo) and the volumetric methane production from manure of different origin can be very variable. For practical purposes, bioprocess efficiency is based on the removal rates of Total Suspended Solids (TSS). This is based on the observation that common wastewater treatment processes are designed to remove TSS and not TS.

Some factors affecting performance are as follows:

A number of factors are important in the operation of an anaerobic digester, including: hydraulic retention time, solids retention time (also known as mean cell residence time), organic loading rate, mixing, pH, alkalinity, temperature, pH, and reactor gas production.

Suggested Operation Parameters for Rural Developing World Applications		
Operation Parameters		Source(s)
SRT min	4	(Tchobanoglous et al., 2003)
lim (d)		
Safety Factor (SF)	10 30	(Rittmann & McCarty, 2001)
SRT (d)	20 70	(Garfi et al., 2011)
pH	6.6 - 7.6	(Tchobanoglous et al., 2003)
OLR (kg VS/(d*m3))	1.0 - 3.5	(Sharma & Pellizzi, 1991)

Hydraulic retention time, θ (days), is defined as the average amount of time one reactor volume of actively digesting sludge stays within the reactor.

$$\theta = \frac{V}{Q}$$

where: θ = hydraulic retention time (d)

V = volume of reactor (m3)

Q = influent flow rate

Where there is no recycling HRT is equal to SRT (Solid Retention Time)

Hydraulic retention time is important to reactor operation and design because it defines the length of time the substrate and particular constituents targeted for removal will be in contact with the biomass within the reactor. Reaction kinetics of methanogenesis and fermentation are the rate-limiting kinetics in anaerobic digestion. Most often, methanogenesis is the rate-limiting step. At temperatures close to 30 ^{o}C, SRT's 20 to 30 days are recommended. It is important to design reactors for sufficient retention times so that volatile solids destruction can take place.

In biological wastewater treatment, large scale reactors are designed with *safety factors* for various reasons, including: the lack of operator oversight, variability of waste water stream, and fluctuations in operating conditions. Safety factors in biological treatment systems are different from safety factors used in structures. The minimum SRT, or the SRT at which washout occurs is multiplied by a safety factor. (Washout is the point at which the growth of the microorganisms contained in the reactor is less than the loss of cells in the reactor effluent. There is a no net loss of cells in the system.) Because the minimum SRT is the borderline of system failure, it is important to have a large safety factor. Specifically, in rural areas of developing countries, there will be fluctuations in various parameters. A Safety Factor of 10 is used in the semi-empirical kinetic model.

Mixing is another important parameter to consider in the design of an anaerobic digester. Mixing increases the rate kinetics of anaerobic digestion, accelerating the biological conversion process. Additionally, mixing allows uniform heating of the reactor. Mixing can be done mechanically through motorized impellers or turbines within the

reactor or pneumatically by injecting gas (in anaerobic digestion, methane and carbon dioxide gas) via spargers at the bottom of the reactor.

The *pH* of the digester is yet another important parameter in anaerobic digestion. The pH should be maintained between 6.6 and 7.6. One difficulty is maintaining pH above 6.6. During digester start-up, overloading, or instability, organic acids are intermediate products produced by the microorganisms. The presence of too high a concentration of organic acids decreases the pH, decreases methane production, and can cause reactor souring or reactor failure. The carbonic acid system dominates pH control most of the time in anaerobic digestion.

Alkalinity is defined as the capacity of water to neutralize acid. In anaerobic digestion, the normal percentage of carbon dioxide in the gas phase is 25 – 45 %.

Because bacterial growth is mediated by a complex set of enzymatic chemical reactions and the reaction rate of all chemical reactions depends on *temperature,* bacterial growth rate depends on temperature. As a general rule, bacterial growth rates double for each 10°C rise in temperature over a temperature range, which varies by bacterial species. For mesophilic anaerobic digestion, the operational temperature range is 10 to 30°C. The operational temperature range for thermophilic anaerobic digestion is 55 to 65°C. Specific methane production rates are 50 to 100 percent higher for thermophilic anaerobic digestion than for mesophilic anaerobic digestion.

Gas production is measured using a flow-through gas meter and used in the calculation of the volumetric gas production (L gas/ L vol of Digester).

❑ *EXPERIMENTAL PROCEDURE*

Set-up of Bench-Scale Digesters

Batch reactors (Digesters1 & 2, each at 30-liter capacity) were assembled from two plastic drums with cover. The PVC stirrer was fabricated having a Ø (diameter) of ½" and ¾" with acrylic sheet (5 mm thick x 2" x 8") connected to a Ø ½" PVC pipe, serving as paddle of the stirrer with gas outlet for each reactor. The two bioreactor-drums were connected in series by a 1/2" pipe. Sampling ports were installed in each reactor with ½" PVC pipes and PV ball valves.

Configuration of a Two-stage Digester and Organic Feed Digestate

During the first-stage, Digester 1 was loaded with organic waste, fresh inoculum and water to a volume of 20 L. The hyrolysis-acidogenesis-acetogenesis reactor, Digester1 was operated as a continuous stirred tank reactor for the Hydraulic Retention Time (HRT) of 6-8 days, at temperature of 34 to 35 ^{o}C, pH of 6.5 to 6.8. For the second stage, Digester 2 was fed with the effluent from the Digester 1 (after 8 days). Digester 2 was "optimized" for methanogenesis at an HRT of 13-16 days, with the same temperature (see Digester 1), at pH of 7.0 – 7.5 and stirred occasionally. Organic feed load of the Digester consisted by weight of: 17% organic swine manure, 30% fresh inoculum, and 53% water.

Determination of Water Quality and Bioprocess parameters

The influent and the effluent samples for each of the Digesters were analyzed for pH, temperature, chemical oxygen demand (COD), biological oxygen demand, total solids, total suspended solids (volatile solids VS), using standard methods (APHA, 1995). COD was measured using the DRB 200 Reactor while BOD_5 was determined using the Oxitap apparatus. Gas volume was measured using a gas meter, Flow Stream® Laminar Mass Flow Meters purchased locally.

Determination of Bioprocess Efficiency

Because common wastewater treatment processes are designed to remove TSS and not TS, total solids in treated effluent generally vary depending on the total solids of the influent. However, treated effluent TS results may actually be greater than influent TS

due to chemical addition in the plant which may add TS to the wastewater. The removal rate or efficiency is calculated as a percentage as follows:

{[Influent - Effluent] / Influent} x 100 = % Removal

Gas Heating Values

We did not experimentally determine the calorific or gas heating values of the biogas produced since we have developed this two-stage digester based on a bioprocess scale. The heating value of a gas is defined as the thermal energy per unit volume of the gas. It is expressed in Btu/ft^3. For natural gas, it is approximately in the range of 900 to 1200 Btu/ft^3. There are two heating values used in the industry. These are the lower heating value (LHV also termed as dry) or the net heating value and higher heating value (HHV also termed as wet) or the gross heating value. For a gas mixture, the term gross heating value is used. It is calculated based upon the heating values of the component gases and their mole fractions. This is the same as the thermodynamic heat of combustion since the enthalpy change for the reaction assumes a common temperature of the compounds before and after combustion. At roughly 60 percent methane, bio-gas possesses an energy content of 600 Btu/ ft^3, which is similar to landfill gas. In Italy most of the biogas is obtained from organic waste present in landfills (Comino et al. 2009). For comparison, please see the table below for energy content of other sources.

Gas	Gross Heating Value		Net Heating Value	
	(Btu/ft3)	(Btu/lb)	(Btu/ft3)	(Btu/lb)
Hydrogen (H2)	325	61,084	275	51,628
Hydrogen Sulphide	672	7,479		
Landfill Gas	476			
Methane - CH4	1,011	23,811	910	21,433
	950	19,500	850	17,500
Natural Gas (typical)	-	-	-	-
	1,150	22,500	1,050	22,000

Gas	Gross Heating Value		Net Heating Value	
	(Btu/ft3)	(Btu/lb)	(Btu/ft3)	(Btu/lb)

Energy Unit Conversion:

1 Btu/ft³ = 8.9 kcal/m³ = 3.73x10⁴ J/m³

1 Btu/lb = 2,326.1 J/kg = 0.55556 kcal/kg

NOTE: A common method of relating HHV to LHV is:
HHV = LHV + {hv *x* (nH2O,out/nfuel,in)}
where hv is the heat of vaporization of water, nH2O,out is the moles of water vaporized and nfuel,in is the number of moles of fuel combusted.

Statistical Analysis

Statistical analyses were performed using available online Statistics and packages such as Kovach Computing Services; Multivariate Analysis for Principal Component Analysis and Clustering. T-test significance was compared with critical values for *t*-test at .05% probability distribution.

❑ *RESULTS AND DISCUSSION*

Tables 1 to 2 showed the results of bioprocess parameters for Reactor 1 and Reactor 2, which were operated in series without recirculation using a fed-batch process.. These parameters were monitored starting November (2012) to June (2013). Using t-test it was shown that % reduction in Total Solids (TS), Total Suspended Solids (TSS), Biochemical Oxygen Demand (BOD) were statistically significant (Please see Table 4). TS was reduced by 69.62%, TSS was reduced by 73.89%, and BOD was reduced by 78.65%; while Chemical Oxygen Demand (COD) was reduced by 56.42%. The average volume of gas production for the second Digester was almost doubled compared to the first Digester although remained non-significant. Figure 1 showed that the gas production was less at the start of the experiment on the months of November and December (2012). This was highest for the month of February and stabilized thereafter.

REACTOR 1								
TIME/ DURATION	PARAMETERS							
	pH	T (^{0}C)	TS (mg/L)	TSS (mg/L)	COD (mg/L)	BOD (mg/L)	Vol of Gas (L)	HRT days
2012								
Nov	6.67	33	8,630	6,340	24,600	8,000	160	7
Dec	6.54	34	10,148	4,400	9,300	5,000	100	7
2013								
Jan	6.5	32	25,133	7,763	11,600	4,500	100	7
Feb	6.5	33	30,928	14,430	9,600	4,250	830	7
March	6.56	34	28,052	13,746	16,700	6,000	700	7
Apr	6.58	35	27,885	12,000	17,800	5,250	630	7
May	6.55	34	16,346.7	7,456.7	10,100	2,000	680	8
Jun	6.81	35	24,916	9,606	18,000	7,500	610	6

Table 1. Bioprocess parameters for Reactor 1.

REACTOR 2								
TIME/ DURATION	PARAMETERS							
	pH	T (^{0}C)	TS (mg/L)	TSS (mg/L)	COD (mg/L)	BOD (mg/L)	Vol of Gas (L)	HRT days
2012								
Nov	7.10	33.8	7,365	3,313	11,000	2,500	460	14
Dec	7.03	33.	8,360	2,323	2,600	500	380	14
2013								
Jan	7.2	33	6,541	3,603	6,700	1,000	340	14
Feb	7.16	35	5,620	1,333	5,000	1,375	1,857	14
March	7.09	35	6,525	3,400	9,600	1,350	1,110	14
Apr	7.05	35	6,883	2,100	6,600	1,300	1,090	15
May	7.07	34	5,498	1,706.7	4,600	700	1,020	16
Jun	7.5	34	5,468	2,000	5,190	350	1,050	16

Table 2. Bioprocess parameters for Reactor 2.

TIME/ DURATION	REACTOR 1	REACTOR 2	**% EFFICIENCY** (REMOVAL RATE) {[Influent - Effluent] / Influent} x 100 = % Removal
	TSS ($\bar{x}$) (mg/L) –INFLUENT-	TSS ($\bar{x}$) (mg/L) –EFFLUENT-	
Nov-Jun	9467.7125	2472.3375	***73.89%***

Table 3. Bioprocess Efficiency.

The bioprocess efficiency of 74 % (Table 3) for the two-stage digestion was calculated from the removal rate of the TSS, based on the literature that common wastewater treatment processes were designed to remove TSS and not TS. (Treated effluent TS results may actually be greater than influent TS due to chemical addition in the plant.)

STATISTICAL SIGNIFICANCE OF BIOPROCESS PARAMETERS					
BIOPROCESS PARAMETERS	**MEAN/ AVERAGE**		**% REDUCTION**	Statistics ***t-test*** Obs	**Statistical Significance** Df=14 .05%; two-sided ***t value*=4.14**
	Reactor1	Reactor2			
Total Solids	21504.8375	6532.5	14972.3375/ $\bar{x}$ ***69.62%***	4.89	*significant*
Total Suspended Solids	9467.7125	2472.3375	6995.375/ $\bar{x}$ ***73.89%***	5.32	*significant*
COD	14712.5	6411.25	8301.25/ $\bar{x}$ ***56.42%***	3.84	*not significant* ***(t** obs lower)*
BOD	5312.5	1134.375	4178.125/ $\bar{x}$ ***78.65%***	5.85	*significant*
Vol Gas (L)	476.25	913.375	***{191.78%***	2.09	*not significant*

			increase}		

Table 4. Statistical Analysis of the Bioprocess Parameters

TIME/ DURATION	Theoretical Maximum Cumulative Methane Gas (ml) [Total Gross]	COD removed (g)	BMP (Theoretical) (ml CH_4/g COD)
Nov	460,000	13.600	33.8235×10^3
Dec	840,000	6.700	125.3731×10^3
Jan	1,180,000	4.900	240.8163×10^3
Feb	3,037,000	4.600	660.2174×10^3
Mar	4,147,000	7.100	584.0845×10^3
Apr	5,237,000	11.200	467.5893×10^3
May	6,257,000	5.500	1137.6364×10^3
Jun	7,307,000	8.30125	880.2289×10^3

Table 5. Biochemical Methane Potential (Theoretical).

The volume of gas production was related to the biochemical methane potential (BMP). This BMP test can determine the methane yield of an organic matter substrate by anaerobic digestion under specific condition and media. It was initially developed in the seventies (Owen et al., 1979). This was based on a common parameter for waste characterization to determine the quantity of methane (ml methane/g COD removed) that the waste can potentially produce in anaerobic conditions. We calculated the theoretical BMP based on the maximum cumulative gas production divided by the amount of Chemical Oxygen Demand for a particular time interval. As shown in Table 5, the theoretical BMP was highest for the month of May 2013. However, this leveled off on the average of the total month-interval for the whole experiment. With 50% methane (Comino et al. 2009), this represented about half of the BMP.

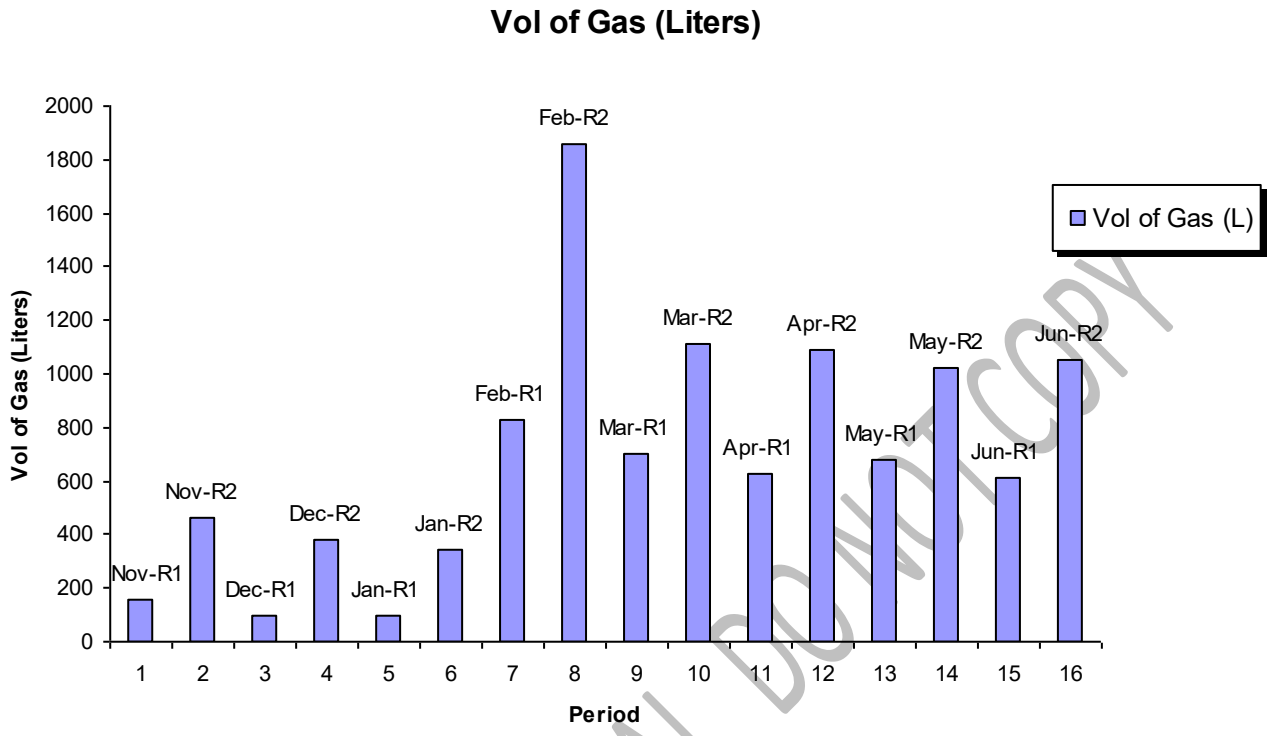


Figure 1. Histogram of Gas Production.

PRINCIPAL COMPONENT ANALYSIS (Log_{10} transformed data; non-centered)			
PCA case scores	**Axis 1**	**Axis 2**	**Axis 3**
pH	1.351	0.072	-0.022
Temperature	2.329	0.095	-0.031
Total Solids	6.137	-0.046	0.175
Total Suspended Solids	5.547	-0.156	0.142
COD	5.99	-0.049	-0.156
BOD	5.073	-0.35	-0.136
Vol of Gas (L)	4.105	0.609	-0.007
Hydraulic Retention Time	1.57	0.253	-0.066

Table 5. Principal Component Analysis.

Principal component analysis (of axis 1) showed the top 5 parameters important to our study namely: Total Solids, Total Suspended solids, COD, BOD, and volume of Gas.

CORRELATION ANALYSIS

CORREL	pH	Temperature	*Total Solids*	*TSS*	*COD*	*BOD*	Vol of Gas (L)	HRT
pH	1.000	0.253	-0.761	-0.771	-0.574	-0.713	0.486	0.911
Temperature	0.253	1.000	-0.129	-0.118	0.009	-0.073	0.640	0.182
Total Solids	-0.761	-0.129	1.000	0.953	0.505	0.631	-0.243	-0.802
TSS	-0.771	-0.118	0.953	1.000	0.621	0.693	-0.262	-0.823
COD	-0.574	0.009	0.505	0.621	1.000	0.911	-0.411	-0.743
BOD	-0.713	-0.073	0.631	0.693	0.911	1.000	-0.473	-0.879
Vol of Gas (L)	0.486	0.640	-0.243	-0.262	-0.411	-0.473	1.000	0.506
HRT	0.911	0.182	-0.802	-0.823	-0.743	-0.879	0.506	1.000

p Values	pH	Temperature	Total Solids	TSS	COD	BOD	Vol of Gas (L)	HRT
Temperature	0.345		0.633	0.663	0.973	0.788	0.008	0.499
Total Solids	0.001	0.633		0.000	0.046	0.009	0.365	0.000
TSS	0.000	0.663	0.000		0.010	0.003	0.327	0.000
COD	0.020	0.973	0.046	0.010		0.000	0.114	0.001
BOD	0.002	0.788	0.009	0.003	0.000		0.065	0.000
Vol of Gas (L)	0.056	0.008	0.365	0.327	0.114	0.065		0.046
HRT	0.000	0.499	0.000	0.000	0.001	0.000	0.046	

Table 6. Correlation Chart

Correlation analysis showed that TS, TSS, COD, and BOD were positively correlated with each other while Vol of gas was negatively correlated.

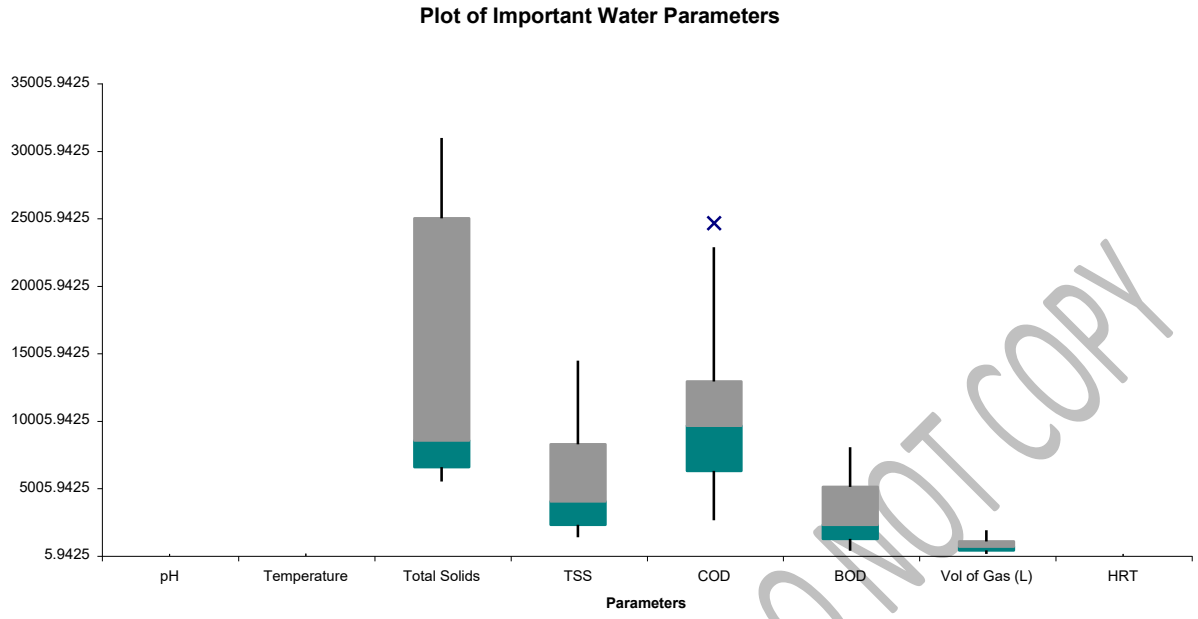


Figure 2. Box-Whiskers plot of important water parameters

The average gas volumetric production rates were 15.875 L/ L Reactor1 or 30.45 L/ L Reactor2. Compared to a pilot-scale of 128L (Comino et al. 2009) (with a vol factor of 4.266) producing biogas of 1919.7 l, which is 50% methane.

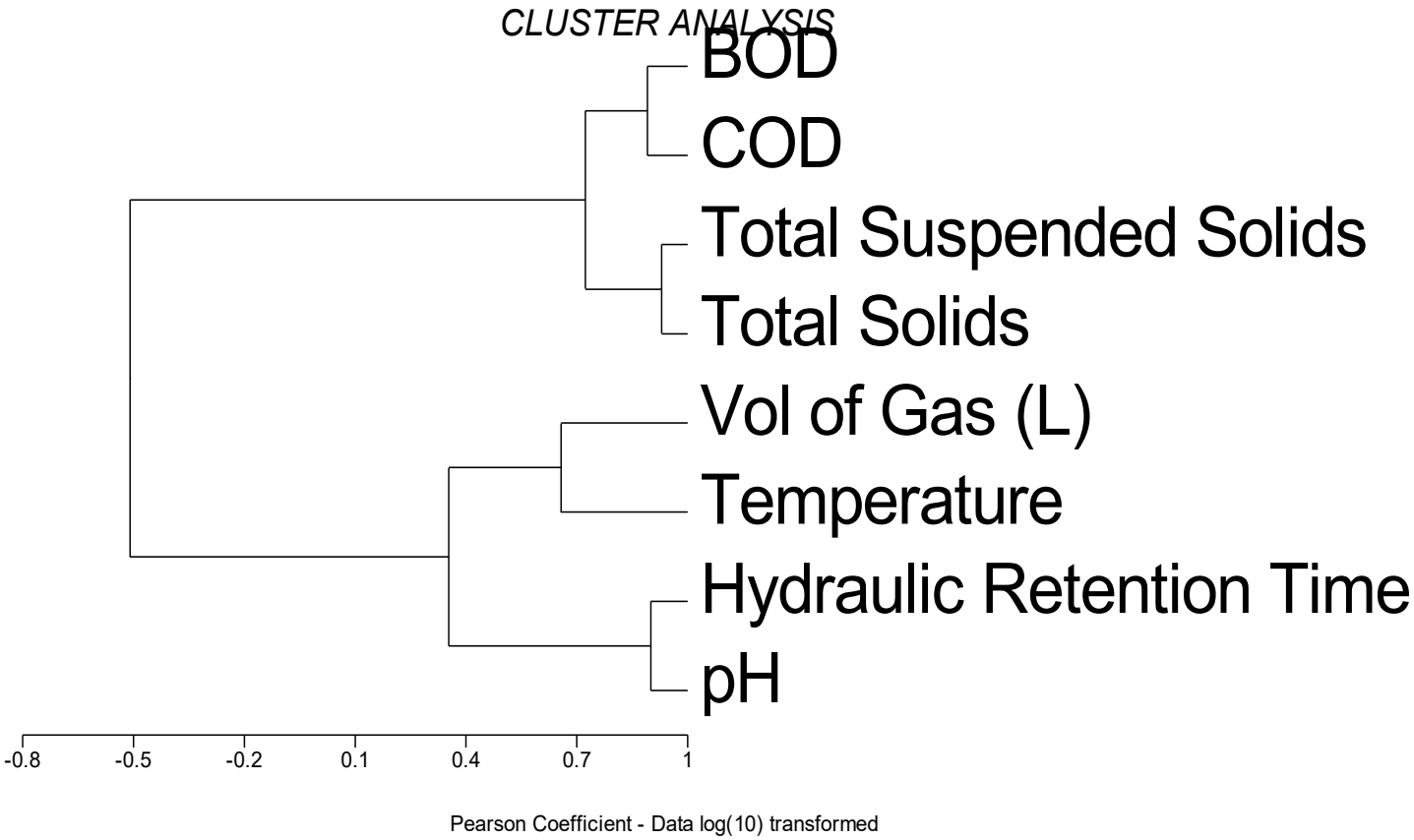


Figure 3. Cluster Analysis with UPGMA

Using "Unweighted Pair Group Method with Arithmetic Mean", (UPGMA) also known as average linkage clustering in Figure 3, data showed that results for BOD, COD, TSS and TS were clustered together, while gas volume, temperature, HRT and pH were clustered as another group.

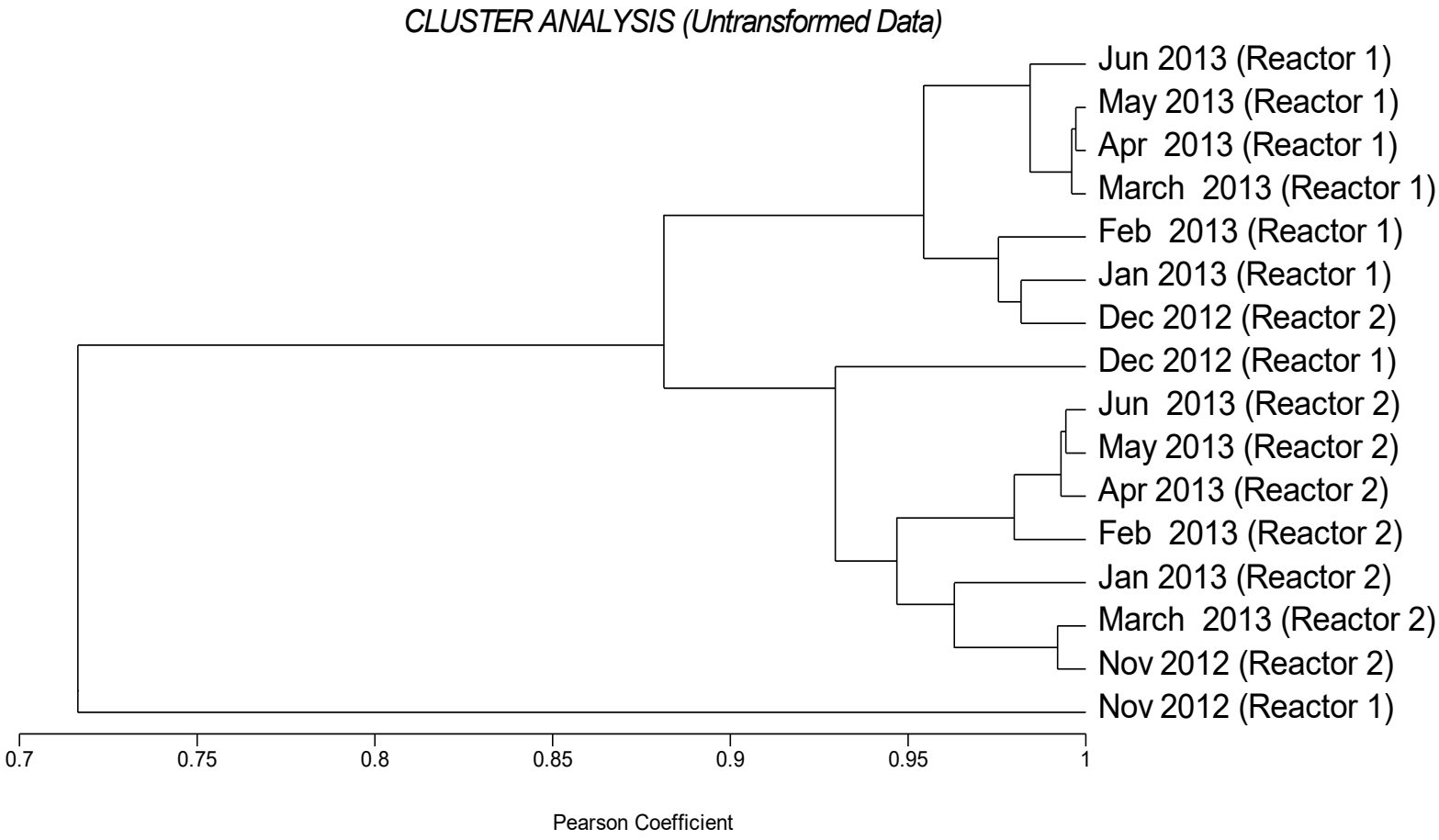


Figure 4. Cluster Analysis without transformation.

Cluster analyses shown in Figure 4 showed that bioprocess data were specific for a given Reactor type: 1 or 2. However, this association was not observed for the data on Nov or Dec 2012. Results for Reactor 1-Nov 2012 is distally connected with the rest of Reactor 1 results, while the parameters results for the month Dec 2012 showed divergence with the Reactor 1 closely clustering with the results for Reactor2; with the Reactor 2 closely clustering with the results for Reactor1.

SUMMARY AND CONCLUSION

The two-stage anaeroabic digestion offered a technological advantage via a threoretical enhancement of methanogenesis, which was delimited at a second digester while precursors, which were primarily volatile fatty acids accumulated at an earlier stage in the first biodigester. This could be applied for specific capture of energy gases such as methane from organic waste. Based on the bioprocess results covering November to June, five important bioprocess parameters were identified using PCA namely: Total Solids, Total Suspended Solids, COD, BOD and Volume of Gas. Statistically significant reduction rates were recorded for TS (69.62%), TSS (73.89%), and BOD (78.65%). These were within range of the baseline values prescribed by the Environment Management Bureau (EMB-DENR), which calls for 90% reduction or 2,000 of BOD for industrial effluent indicators. However, the effluent cannot be directly discharged to bodies of water due to pervasive TS, TSS, COD levels. These parameters were positively correlated while the volume of gas was negatively correlated since the volume increased twice compared to the first digester. Data clustering analysis showed that the data obtained from Digester 1 were distinct from the data gathered from Digester 2. The volumetric gas production were stable and increased especially on the first quarter of 2013. Our bench-scale two-stage process exhibited an efficiency of 73.9%. A theoretical biochemical methane potential was calculated based on the maximal

cumulative methane gas volumes of Reactor 2, which represented only half of this value for methane.

- *TECHNO-ECONOMIC DATA:*

Eco-technology drivers were favored in our technology to support green energy production and at the same time countered the effect of climate change; while converting waste into useful energy.

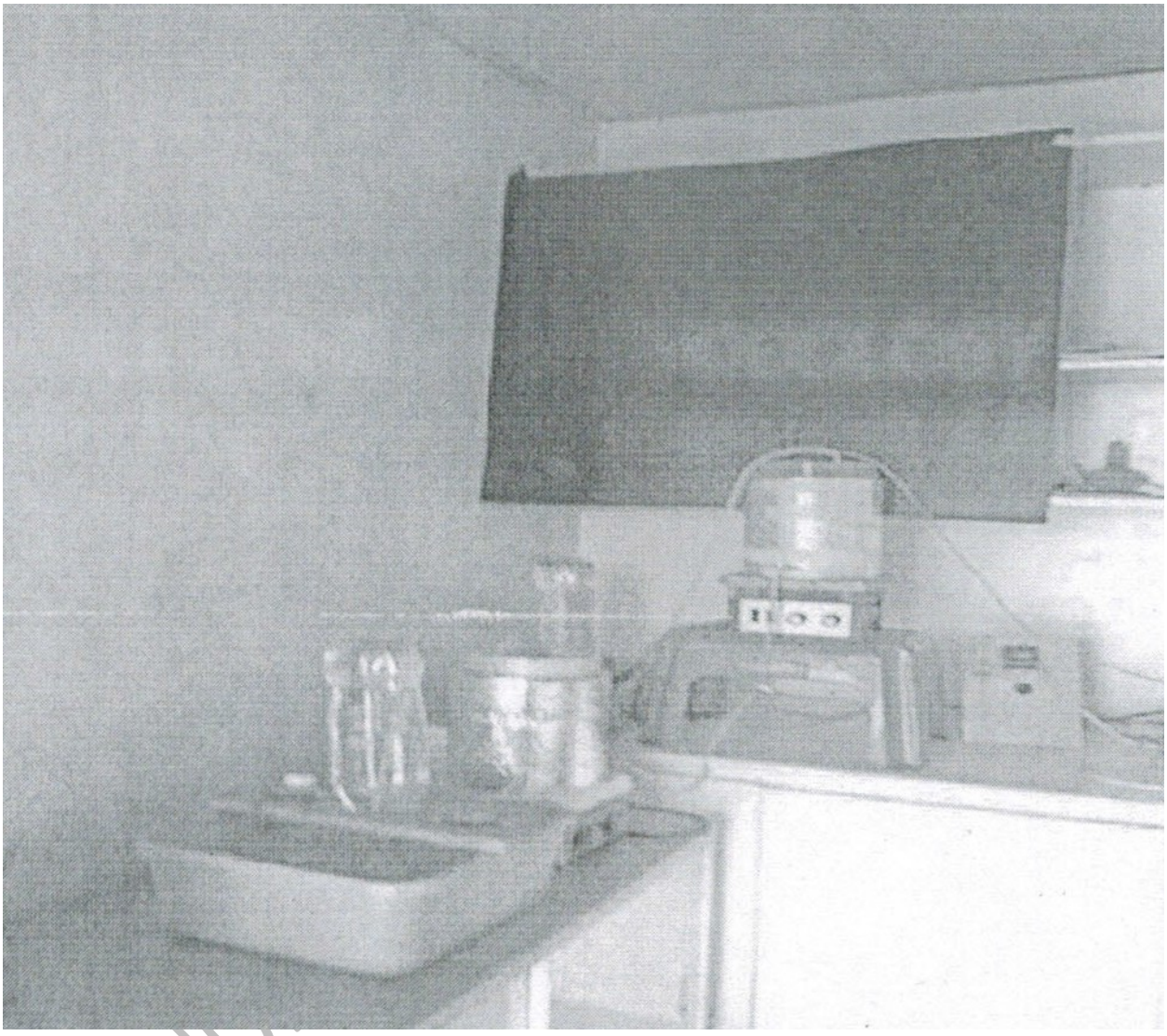
- *SOCIO-ECONOMIC DATA:*

The described two-stage bioprocess technology could enhance social benefits such as in providing threshold incentives and livehood to the rural community as well as a clean healthy environment since hazardous waste organic animal manure could be used as a raw material in this technology.

- *RECOMMENDATION*

We recommend the determination of calorific or gas heating values in order to assess the energy conversion as applied to electricity generation, boilers, combined heat and power, etc.

PICTURES



Two-Gallon Reactors for Two-stage Anaerobic Digester

30-L Batch Reactors for 2-stage Process

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CHAPTER 3

MICROBIAL PROCESSING OF NATURAL RUBBER WASTE

NOEL M. UNCIANO

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ABSTRACT

This paper points on the importance of research for competent microbial isolates for the development of microbial process for the recycling and treatment of waste rubber materials. Microbial flora are found in many latex rubber wastewater effluent representing an untapped resource, which could harbor important functions in bioremediation. In the ASEAN region, a sizeable sink of spent raw materials are extruded in the output of rubber processing. Reports in Malaysia showed that approximately 20 tons of rubber and 410 thousand litres of effluent per day are produced by the rubber factory. This study provides background and suggested methods for studying microbial flora for conversion of rubber waste stream.

SIGNIFICANCE

This research study is geared towards the isolation, as well as the characterization of competent microbial isolates for the development of microbial process for the recycling and treatment of waste rubber materials. The primary sources of isolates are soil at locations in rubber producing plants, latex processing residues or leftovers various tissues from rubber plants or amendments via enrichment procedures of collected polluted local soil, effluent, and environmental samples from various sites with latex waste residues. Since rubber waste biodegradation involves gaseous by-products, which are difficult to characterize, the Denaturing Gradient Gel Electrophoresis and quantitative RT PCR will be used to detect genes active during the microbial processing. Process treatment with microbial isolated biomass will be conducted in bench-scale application to obtain recycling with the recovery of isoprene and co-products. Bench-scale treatment of wastewater from primary processing of rubber will likewise be considered in order to reduce their environmental impacts.

The use of bacterial strains such as the purple nonsulfur photosynthetic bacteria (PNSB), *Rhodopsuedomonas* is a favorable technology which is ecologically and environmentally promising since better than 90% of Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) in treated rubber wastewater effluent could be reduced by these organisms. The treatment of latex centrifugation effluent with free cells of *Bacillus* sp. SBS25 showed 68% reduction in COD while treatment method in an activated sludge reactor using the consortia and the SBS25 isolate resulted in 92.5% reduction (Cheriana and Jayachandrana 2010). Microbial flora are also found in many latex rubber wastewater effluent representing an untapped resource, which could harbor important functions in bioremediation.

A recent paper reported the use of quantitative real-time PCR for assessing water quality in landfills in correlation with BOD/ COD associated microbial gene expression (Han and Kim 2009).

In the ASEAN region, a sizeable sink of spent raw materials are extruded in the output of rubber processing. Latex waste residues accounted to about 12% of the total daily effluent of a Vietnamese natural rubber factory. In local terms, this translates to about 30,000 kg/ day assuming a minimal 10%. Reports in Malaysia showed that approximately 20 tons of rubber and 410 thousand litres of effluent per day are produced by the rubber factory.

THE EMERGENCE OF THE BIOECONOMY

The twenty-first century has been characterized by the emergence of new challenges faced by globalization amidst the need for new socioeconomic and resource scarcity caught by rapid urbanization and population surge, environmental protection and regulation, an expanding global class hungry for automobiles and modern technology, and more volatile finances that face the global market. The term Bioeconomy was the product of these global mosaic of challenges. What Golden & Handfield (2014) had put it, Bioeconomy is global industrial transition of sustainably utilizing renewable aquatic and terrestrial resources in energy, intermediate, and final products for economic, environmental, social, and national security benefits. The White House (2012), declared "bioeconomy is one based on the use of research and innovation in the biological sciences to create economic activity and public benefit." Quoted in the Organization for Economic Co-operation and Development (OECD 2009): "From a broad economic perspective, the bioeconomy refers to the set of economic activities relating to the invention, development, production and use of biological products and processes. If it continues on course, the bioeconomy could make major socioeconomic contributions in OECD and non-OECD countries. These benefits are expected to

improve health outcomes, boost the productivity of agriculture and industrial processes, and enhance environmental sustainability."

TECHNICAL JUSTIFICATION

The assessment of aquatic ecology has relied most commonly on the macro invertebrates as indicators. However, bacteria and other microorganisms may also be informative of the condition of aquatic ecosystems (Wakelin et al. 2008) and could thus be considered as environmental bioindicators. These are common biota in sediments and biofilms, in the water column but also are also ubiquitously present at high abundance in aquatic systems. However, much research on microbial bioindicators in aquatic systems has been limited to heterotrophic bacteria in relation to the decomposition of dissolved organic matter (Geldreich, E. E. 1976) and as a measure of sewage pollution (Miescier, J. J., and V. J. Cabelli. 1982). Technical understanding of basic processes and their relation with environment is clearly sought after since the microbial interaction is based on the function of genes and this will contribute to our understanding of microbial bioindicators, which has been quite limited as stated.

Molecular characterization including the sequencing of specific bands in the DGGE will then constitute a major effort and has to be included in the screening objective and in objective #5. This is perceived in an apriori premise that the information of genes and their sequences is a basic information and is inclusive in the screening objective. This is inherent in studying the distribution as well as the dynamic structure of microbial communities with the functional niche (as to where in the environment they could function), which will help us achieve environmental efficiency.

Environmental protection is grounded in to basic principles: Principle 4, Rio Declaration on Environment and Development and the International Declaration on Cleaner Protection.

Thus, there is a pressing need to study and implement wastewater treatment in the country. Based on a recent study (Water and Environment Partnership in Asia WEPA 2012) among non-pollution sources, agricultural runoff is the major source at 74% in terms of BOD. According to a recent report (ADB, Urbanization in Asia, 2011) Most Asian cities do not have effective wastewater treatment systems. In the Philippines, for example, only 10% of wastewater is treated while in Indonesia the figure is 14%, in Viet Nam, 4%, and in India, 9%. As published in the WEPA report 2012 the country experienced increasing trends for BOD levels as observed in many priority rivers (525 bodies of water) some of which exceed 300% in BOD levels in 2010 (compared to 2003 levels). Based on the data of a commercial website, surface water is the major source (at 73%) of potable water in the urban population.

Pollution is constantly eroding our water resources and could have adversely affect the health of the population in the long run; such as the prevalence of antibiotic resistance genes (ARGs). Even subtoxic levels of zinc, which is used in the rubber latex processing could cause induced antibiotic resistance. Stoll et al. 2012 reported the wide distribution of ARGs for sulfonamide, trimethoprim, macroline, β-lactams and chloramphenicol in the aquatic ecosystems, which serve as the reservoir of ARGs genes and could potentially be transferred from commensal microorganisms to human pathogens; furthermore the ARGs have been found to be resistant to UV irradiation.

OBJECTIVES

The study aims to develop microbial process for the recycling and treatment of wastes from the primary processing activities of rubber.

Specifically, the project shall have the following objectives:

1. Isolate microbial strains for the degradation of wastes from rubber primary processing
2. Screen microbial strains for waste degradation properties and translucent halo formation in agar-latex media plates
3. Screen microbial strains for extra-cellular protease activity and clearing zone formation in gelatin/ or casein
4. Characterize microbial isolates and assess the potential application in rubber waste recycling and treatment
5. Characterize microbial processing using Denaturing Gradient Gel Electrophoresis and PCR
6. Conduct bench-scale rubber waste processing/ wastewater treatment using microbial isolates

LITERATURE

Technical with Economic Justification

A report assumed that in Vietnam about 80 percent of the rubber plants use open lagoons for wastewater treatment (Resource Assessment Report for Livestock and Agro-Industrial Wastes – Vietnam 2010). In the South East region, treatments are based on conventional biological processes, for example: pond system, oxidation ditch, anaerobic digestion and activated sludge. However, based on current literature (Nguyen & Loung 2012), these processes have not sufficiently met the quality requirement for the effluent of natural rubber processing industry in Vietnam (QCVN 01:2008/BTNMT). Moreover, there has been no implementation of a full combination of the biological, physical and chemical processes. Technical treatment processes in rubber factories in this region are shown in Table 2.

Table 2. The average capacity of influent and the technical processes for wastewater treatment system of some rubber processing factories in South East region, Vietnam

No.	Factory	Company	Average volume capacity of influent (m^3/day) *		Technical process for wastewater treatment of the processing of concentrated latex rubber **
			Concentrated latex wastewater	Other kinds of wastewater (miscella-neous latex, SVR3L, SVR 10)	
1	Loc Hiep	Loc Ninh One Member Co., Ltd	450	550	Decantation - UASB – aera-tion tank – settling and filter
2	Quan Loi	Binh Long Rubber Company	500	-	Decantation – oxidation ditch – settling and filter
3	Tan Lap	Dong Phu Rubber Joint Stock Company	300	-	Decantation – oxidation ditch – settling and filter
4	Tan Bien	Tan Bien One Member Co., Ltd	300	700	Decantation – oxidation ditch – settling and filter
5	Ven Ven	Tay Ninh Rubber Joint Stock Company	550	950	Decantation – flotation – oxidation ditch – settling and filter
6	Bo La	Phuoc Hoa Rubber Joint Stock Company	400	-	Decantation – flotation – UASB – aeration tank – set-tling and filter
7	Xuan Lap	Dong Nai Rubber Coporation	700	1,000	Decantation – oxidation ditch – settling and filter

* The average data were calculated during November, 2011
** Source: Conference of the summarization of mechatronics, processing and environment of Vietnam Rubber Group, 2009

The technology of membrane bioreactor (MBR) can overcome the disadvantages of biological methods. According to the study (Nik et al 2010), the process could be continuously operated for more than one month without the chemical cleaning of membranes. The removal efficiency of COD was 96.99% at the initial concentration of 3,500 mg/L COD, of BOD was 96.78%, total-N 65.17% and N-NH3 61.35%.

In Malaysia, aerobic and anaerobic treatments are the most common biological method used for treating rubber wastewater with high efficiency, low in capital costs but required land space. In some rubber factories where land area is limited, aeration systems are used as an alternative of settling ponds. The best novel methods also are shown in Table 4 (Mohammadi et al. 2010).

Table 4. Various rubber wastewater treatment systems and their efficiency

Treatment	Description	Initial COD (mg/L)	Initial BOD (mg/L)	Initial TKN (mg/L)	Initial sulphide (mg/L)	COD removal efficiency (%)	BOD removal efficiency (%)	TKN removal efficiency (%)	Sulphate removal efficiency (%)	SS removal efficiency (%)	Reference
Conventional/Current technologies											
Anaerobic filter.	Packed with aquarium media with dimension of 30 cm *100 cm; OLR = 11.8 $gCODL^{-1}$ day^{-1} and HRT = 10 days.	18219	12750	-	-	92	-	-	-	-	Anotai et al. (2007)
Up-flow anaerobic sludge blanket (UASB).	Steel cylinder shape with dimension of 600 m^3 *250 m^3, consists of a waste water distributor, a lid for scraping sludge, dry rubber content and a gas-solids separator.	6100	-	315	-	80	-	80	90	-	Taechapatarakul (2008)
Biological method incorporated with sulphate reduction system (purple non-sulphur photosynthetic bacteria).	Optimum growth in latex rubber sheet wastewater with 0.50% ammonium sulphate and 1 mg/l nicotinic acid in a pure culture and or a mixed culture.	7328	4967	-	-	90	90	-	92 - 96	-	Kantachote et al. (2005)

Several types of enclosed anaerobic digesters were evaluated; however this resulted in frequent clogging of biomass in the packed bed system. This led to the development of anaerobic sludge blanket reactor (UASB), which can control the fault smells emission from the oxidation and stabilization ponds. However difficulties remain in developing the granular sludge blanket and maintaining its stability. However, this system was used in many industries without the legal procedures that this could be appropriate for the treatment of natural rubber wastewater.

An essential difference between anaerobic and aerobic wastewater treatment systems is that the loading rates of anaerobic reactors generally are not limited by the supply of any single reagent, like oxygen in aerobic systems. During the last three decades several high rate anaerobic reactors configurations have been developed.

A study (Jawjit and Lliengcharernsit 2010) indicated that the application of the two-stage upflow anaerobic sludge blanket UASB to concentrated latex processing wastewater is feasible. Nevertheless, combination with other treatment systems (e.g., oxidation pond, aerated lagoon) is necessary to meet Thailand's industrial effluent standards (in the case of COD). Using the Hydraulic Retention Time (HRT) at 24 h and 48 h (optimal

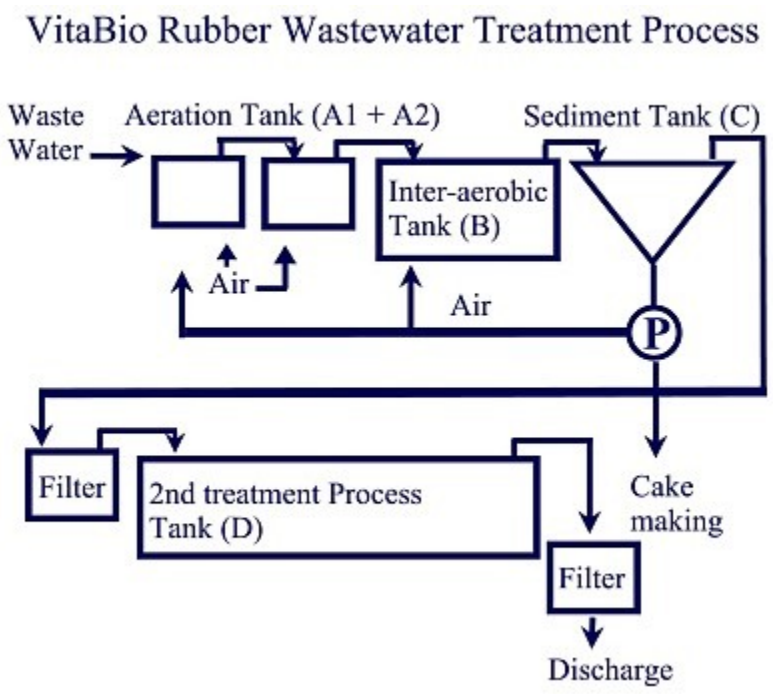
HRT for the acid tank and the UASB tank, respectively) resulted in a reduction of 82% for chemical oxygen demand (COD).

In a recent report (Tanikawa et al. 2012), treated natural-rubber latex wastewater containing a high concentration of sulfate using a combined system consisting of a two stage reactors of up-flow anaerobic sludge blanket (UASB) and a down-flow hanging sponge (DHS) reactor as a post treatment for a period of 10 months and maintained an 11.1 days of the hydraulic retention time however recycling was needed to improve the COD reduction beyond 70.2%. In terms of power consumption, the two stage system was 93% less than that of the conventional lagoon system, reduced the amount of excess sludge discharged by 90%, and reduced the GHG emissions from the aerated lagoons by 95% by recovering methane.

In another report (Phoolphundh et al. 2013) using a two-stage upflow anaerobic sludge blanket reactor results showed a relatively high-rate treatment for latex-processing wastewater having a hydraulic retention time of about 2.5 days and the removal efficiencies of the system were 51.6 % (COD) and 75.9% (sulfate). Monitoring the microbial diversity with DGGE revealed that there was more sulfate-reducing bacteria in both reactors than *archea* bacteria.

In a recent publication, Nurul Zaizuliana R. A. et al. (2013) used an effective microbial technology in Anaerobic Sequencing Batch Reactor (ASBR) system to treat rubber processing wastewater. Pollutants concentrations were reduced to 60% of COD and 62% of BOD5 reductions.

The VitaBIO Treatment Process, features a phasic Inter-aerobic Tank (B), which could periodically function under (micro)anaerobic and aerobic conditions depending on the amount of oxygen pumped into the system.



The major steps of Rubber Wastewater Treatment Process of VitaBio:

1. Aeration Tank Operation (A1 +A2)

This stage oxidizes and reduces the concentration of chemical preservatives to become biologically degradable. The pump-aerated wastewater initially is treated in the Tank A1, and the flow-over water is continuously treated in the Tank A2. Some of the formalin materials are oxidized, and some of the formalin materials are absorbed by the sludge in the aeration tank.

2. Inter-Aerobic Operation (B)

This stage consumes the major portions of organic chemicals in the wastewater. In this inter-aerobic tank (or also called intermittence-aeration tank), the amount of oxygen supply is controlled. Because under the low dissolved oxygen condition, when aeration operation is closed, the remained oxygen in the water will be consumed in a short period of time, therefore the tank which was original in aerobic operation can turn into

anaerobic operation within short time frame. Through the periodical aerobic and anaerobic operation, the microorganisms “digest” the chemicals and/or release them while the decomposition speed can be greatly improved. Thus this operation procedure can have much better effective performance than single aerobic or single anaerobic operation procedure, and it also shows better efficiency.

3. Sediment Tank Operation

This stage collects solid materials through sedimentation process and provide activated sludge to inter-aerobic tank and aeration tank. These sludge is recycled back into the inter-aerobic tank for further process and the extra sludge also can pump into the aeration tank for the oxidization process.

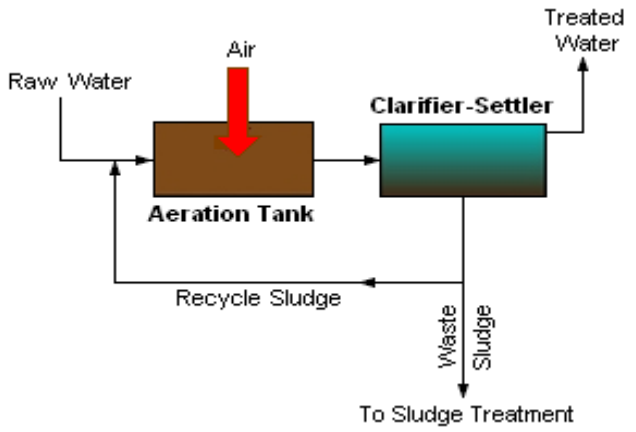
4. 2nd Treatment Operation

This stage performs inter-aerobic operation in a longer resident time. The wastewater from sediment tank passes through the screen filter and enters into the 2nd treatment operation. By controlling the amount of oxygen supply, the tank can be in the mode of aerobic and anaerobic operation sequentially. After this treatment, the water passes through a screen filter for discharge.

For high COD content, the aeration in inter-aerobic tank operates 40 to 50 min., and shut off 10 to 20 min. For low COD content, the aeration of inter-aerobic tank operates 10 to 20 min., and shut off 40 to 50 min. Dissolved Oxygen maintenance range is between 0.3 ~ 2.5 mg/l, the preferred range is between 0.3 ~ 0.7 mg/l. The total capacity of activated sludge is about 5% ~ 10% of the size of inter-aerobic tank, in order to maintain the biological cycling period between 24 to 48 hours. The amount of activated sludge in the activated sludge tank is about 30% ~ 40% range.

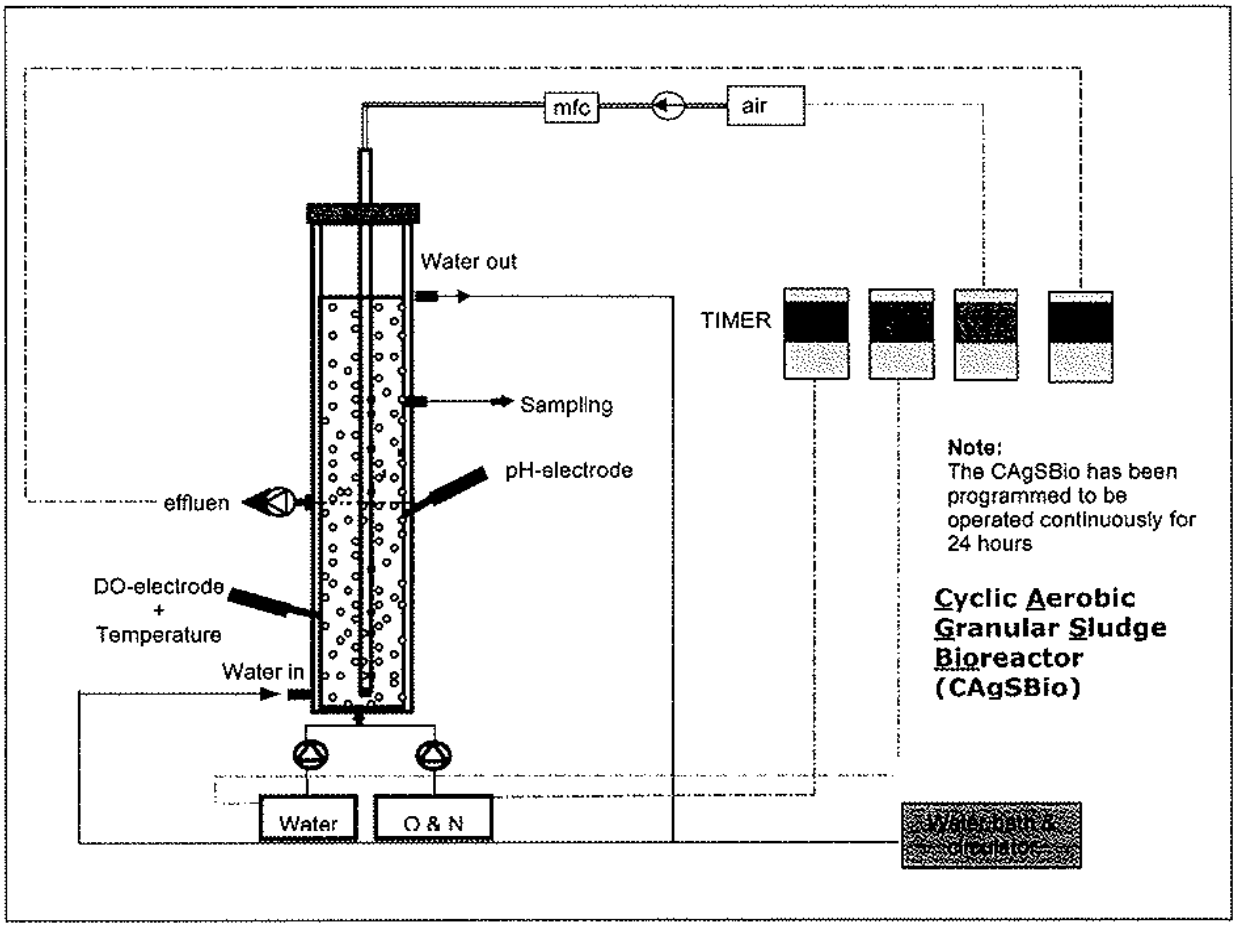
The activated sludge is a process in waste water treatment in which air or oxygen is forced into waste water liquor to develop a biological flock which reduces the organic content of the waste water.

This process is used by the Bridgestone Sumatra Rubber Estate (BSRE) at Medan, Indonesia.



The activated sludge technology requires large footprint (big settling tank) due to the relatively slow settling characteristics of sludge flocs. In contrast, the Aerobic Granular Sludge (AGS) technology offers a possibility to design a compact system based on simultaneous organic and nutrient removal and because of the good settling characteristics of the AGS, the use of a big settling tank is not necessary. Thus, the

AGS installation can be cheaper and more compact. This has been studied to improve sludge settling and behaviour in activated sludge systems. The main advantage is that aerobic granular sludge (AGS) can settle very fast in a reactor or clarifier because AGS is compact and has strong structure. It also has good settleability and a high capacity for biomass retention. This has been used by Rosman et al. (2012), for rubber wastewater treatment using sequential batch reactor with a cycle time of 3 hr using a single column reactor. However the system requires regulated temperature and pH (27±1°C and pH 7.0±1). In this system similar physical properties could be developed in single reactors however, different nutrient elimination performances and microbial communities are affected by temperature.



3L CAgSBio Cyclic Aerobic Granular Sludge Bioreactor on 24-hr continuous operation. (Nor-Anuar 2008 Institute of Environment and Water Resource Management (IPASA), Universiti Teknologi Malaysia).

A new technology, swim-bed combined with aerobic granular sludge (Zhang et al. 2007) was developed for wastewater treatment on the basis of the biofilm process and activated sludge process, and results demonstrated notable performance of high-efficiency treatment capability and sludge reduction. The hydraulic retention time (HRT) was only at 3.2 h. The results showed that COD removal and nitrification efficiency were high at the volumetric loading rates (VLRs) equal and less than 1.0 kg COD/m³/d, corresponding to 0.13 kg N/m³/d. COD removal and nitrification efficiency were above 90% and 73%, respectively using an acryl-fibre biomass carrier (biofringe) for the treatment of latex wastewater (Le, Nguyen et al. 2012).

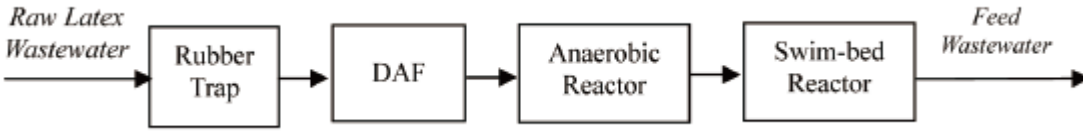


Diagram of pretreatment of feed wastewater effluent with a swim-bed reactor.

Aerobic granulation constitutes a novel technology, which was recently developed (Beun et al. 1999; Tay et al. 2001). This process is a microbial cell self-immobilization forming microbial aggregates that have a strong and compact structure, consisting mainly of aerobic and facultative bacteria and is distinct from anaerobic granular methanogenic sludge. The settling velocity and density of aerobic granules are much higher than those of conventional bioflocs, while the granules have a large surface area and high porosity and have been demonstrated for treatment of a wide variety of wastewaters. Stable aerobic granules could be cultivated in substrate with high levels of ammonium salts that could stably exist for 216 days in continuous-flow reactors with

or without submerged membrane (Juang YC; Adav SS et al. 2010). Single strain dominant (*Candida tropicalis*) aerobic granules have been observed in studies of phenol degradation showing that primary microbial strains could enhance degradation of pollutants (Adav SS; Chen MY et al. 2007). Stable aerobic granular sludge could be developed in an SBR (Sequencing Batch Reactor) for palm oil mill effluent. Following granulation, good accumulation of biomass in the reactor and good settling characteristics were observed. The granules showed results of 91.1%, 97.6% elimination rates for COD and ammonia respectively (Abdullah et al. 2011).

Using a strain isolate, *Bacillus* sp. SBS25 with native consortia flora (Cherian, and Jayachandran 2010) in an activated sludge system had reduced the pollutants to 92.5% of COD. The sole strain isolated contributed about 68% reduction. A set up of aerobic biofilm reactor in H_2S removal were assessed by Chaiprapat S et al. 2011. It was found that H_2S removal efficiency increased with increasing air mix ratio and retention time (RT) with the average removals of 94.7% at 160 RT under a 1:4 biogas-to-air ratio with the acidic biofiltration conditions. This technology research could turn to the a development of efficient and low-cost metal absorbents for cadmium, cupper, zinc (Liu et al. 2002) which are often associated with wastewater from natural rubber latex.

In Sri Lanka based on 10-year study (Vithanage 2003), mechanical aerated lagoon system was more cost effective wastewater treatment process for natural rubber industry where the land is available. However, the initial capital cost of the activated sludge process at Parakaduwa Factory was lower with aeration tank and a sand bed.

According to cost estimates (Lehmonen 2012, Thesis), the use of membranes would be more expensive than oxidation or adsorption treatment, but the estimates are not comparable to each other, because some of the calculations only include the operational costs and some other also include the investment costs and calculations are

based on different size wastewater volumes. Thus, based on these estimated costs, it is not possible to say which of these technologies are the most economical. When advanced oxidation processes are viewed, it can be said that the use of H2O2 increases the treatment costs. In addition, cost efficiency of different technologies in addition to the costs treatment efficiency must also be taken into consideration.

The configuration of a working batch reactor for treatment of rubber latex wastewater will be designed based on the use of cultivated isolates as monocultures or in combination with the normal flora:

i) built in an activated sludge setup

ii) built in a granular sludge setup

iii) built with a combination of a biofilm/or biomembrane setup

The wastewater footprint for natural rubber processing is considerably large. For the production of one ton of rubber about 150 cubic meter of wastewater is produced compared to 10-20 cubic meters in the processing of an equivalent amount of fruits. The effluent liquor discharged directly from the natural rubber process has very high concentration of Biological Oxygen Demand (BOD of 5,000 – 6,000 mg/l) and Chemical Oxygen Demand (COD of 9,500 – 12,000 mg/l) for the centrifuge process, which also contains significant amount of ammonia nitrogen.

Based on the studies in the upstream intermediate natural rubber production in the ASEAN region, a sizeable sink of spent raw materials are extruded in the output of rubber processing. Latex waste residues accounted to about 12% of the total daily effluent of a Vietnamese natural rubber factory.

In Malaysia, rubber wastewater contains considerable amounts of skim, latex serum, uncoagulated latex and washings from the various processing stages. Approximately

20 tons of rubber and 410 thousand litres of effluent per day are produced by the rubber factory. However, reports in several studies showed daily discharge of about 80 million litres of untreated rubber effluent to nearby streams and rivers. The high concentration of nitrogen (including ammonia-nitrogen), sulphate, and heavy metals such as zinc, copper, and cadmium, pose a threat to the environment.

Microbial treatment of latex wastewater is currently limited by low viability due to high organic load and high ammonia concentration severely affects the anaerobic process. Odor and gaseous pollution are mitigated using various solid scrubbers but are also a major environmental issue since this could affect water palatability. Gaseous emission from the residual latex could be considered as a renewable source of added raw materials, which could be enhanced or processed by synergistic microorganisms.

Anaerobic digestion is an attractive waste treatment practice in which both pollution control and energy recovery can be achieved. However, the inhibitors commonly present in anaerobic digesters such ammonia, sulfide, light metal ions, heavy metals, and organics tend to be concentrated along the latex wastewater streams. The use of Coconut shell fibre in anaerobic and aerobic processes has encountered difficulties such as clogging, which necessitated a filtration step before treatment. With the development of anaerobic sludge blanket reactor (UASB), high organic loading could be achieved in the digester. The main difficulty with the UASB lies in developing the granular sludge blanket and maintaining its stability.

The use of bacterial strains in rubber wastewater treatment is considered as an ecologically and environmentally favourable technology. *Rhodopseudomonas* DK6 isolated by Kantachote et al. 2005 showed the best potential for effluent treatment since it can grow well under microaerobic-light conditions and a mixed culture. This purple nonsulphur photosynthetic bacteria (PNSB) from rubber sheet wastewater (in

Thailand) grows optimum in a mixture of 0.50% ammonium sulphate and 1mg/l nicotinic acid with latex rubber sheet wastewater. A 90% reduction of COD and BOD concentrations have been reported with this PNSB isolate. In other treatment regimes such as in sewage wastewater, selected microbial consortia comprising *Bacillus pumilus, Greviacterium* sp, and *Pseudomonas aeruginosa* could effect percentage degradation of 79% for COD and 85.5% for BOD in 4 h incubation (Dhall P et al. 2012).

The EMMC technology or the entrapped mixed microbial cell technology is a system that entraps the mixed microbial cells into the polymeric carriers; membrane bioreactor (MBR) consisting of membrane sheets/fibers to effectively retain the biomass in the reactor. These two biotechnologies increase the solid retention time (SRT) and are thus able to retain high concentration of biomass in the reactor. A membrane bioreactor consisting of two Kubota flat sheet membranes (pore size 0.4 um), with biomass acclimatization, has been used to treat latex wastewater. The BOD and COD removal efficiencies were 96.78% and 96.99%, respectively. This in technical aspects would suggest that entrapped mixed microbial cell (EMMC) technology could considerably enhance the efficacy of the system using microbial strains. Recent technologies using expanded bed biofilm reactor and sequencing batch biofilm reactor results showed that heavy metal adsorption by these reactors are 50 - 95%.

Several procedures consisting of physical processing, co-digestion with other waste, adaptation of microorganisms to inhibitory substances, and incorporation of methods to remove or counteract toxicants before anaerobic/ aerobic treatment can significantly improve the waste treatment efficiency.

Other components present in natural rubber (NR) latex, such as proteins and phospholipids have been shown to be associated at the rubber particle surface. These phospholipid-protein layers are important in the colloidal stability of the NR latex. The

presence of protein degrading activity during the upstream processing of natural rubber will be addressed using samples from various processing steps to be able identify prospective hotspots of microbial biodegradation of latex and thereby improve on the processing recovery.

In the processing of skim latex, trypsin is used to further increase the rubber content (and remove the proteins). The quantity of the enzyme used is however limited to 0.5% since allowable protein content must not exceed 3.1% in the finished product. Sourcing a microbial process for the removal of proteins from latex wastewater (containing latex residues) would present a biotechnological advantage for scavenging latex from wastewater. This procedure could be used in combination with the process of assisted biological coagulation (ABC), in which the microbial growth necessary for the production of acid is accelerated by adding sugar.

THE BIOTECHNOLOGICAL SIGNIFICANCE OF ISOPRENE

Bio-isoprene accounts to as much as 27% of the contents of new tires (Biofuels Digest 4 May 2010), while one liter of petrochemically derived isoprene requires about 7 liters of crude oil. Thus, the raw materials for bio-isoprene from natural products (plant, microbes, or biomass derived) have the potential for reducing (GHG) emissions.

Tire rubber usually consists of 40 to 50 percent rubber (styrene-butadiene rubber, natural rubber, and butyl rubber), 25 to 40 percent carbon black, and 10 to15 percent low-molecular-weight additives. The exact composition depends on the type of tire and the design process of the individual tire manufacturer. ADVAC Elastomers, Inc.

reported that it has successfully developed a proprietary product (TIRECYCLETM) which can be blended with virgin rubber and contains up to 87 percent recycled content.

Isoprene (boiling point, 34 ^{0}C) is a gas at low temperatures and bubbles out of the fermentation process in the gas phase thereby ameliorating costly downstream processing. However, bio-isoprene has a better environmental performance than synthetic rubber, but neither is biodegradable under natural conditions. According to Goodyear, the "BioIsoprene™ product will serve as a renewable and cost-competitive alternative to isoprene since it could provide a hedge against rising crude oil prices".

THE MICROBIAL CHALLENGE

At the turn of the century developing renewable biofuels will be a key factor to meet global demands for energy and synthetic chemistry feedstock without effecting climate change and environmental complications.

Based on a news report of Chemical & Engineering News (Dec 12, 2011), microbial fermentation holds promise for making three renewable rubber intermediates: isoprene, isobutene, and butadiene. The demand for the five- and the four-carbon products will continue to rise in the near future. However these processes have to be cost competitive with the petrochemical pathways. The enzyme isoprene synthase has been identified in plants and through synthetic biology its expression has been optimized in several microorganisms.

Waste microbial degradation involves a number of microbial communities and the by products of rubber, polyisoprene are gaseous in nature and are difficult to characterize.

The basic molecular mechanism by which rubber is degraded is not known. Tsuchi and coworkers were the first researchers to isolate and identify low-molecular-mass

oligo(cis-1,4-isoprene) derivatives with aldehyde and keto end groups from rubber-grown cultures of *Xanthomonas* and *Nocardia* species. Several Actinomycetes isolates of the genus *Nocardia* have been shown to degrade trans-polyisoprene. The trans-isomer of polyisoprene [poly (trans 1-4 isoprene)] found in the plant, Gutta-percha, is being used for several technical applications due to its resistance to biological degradation.

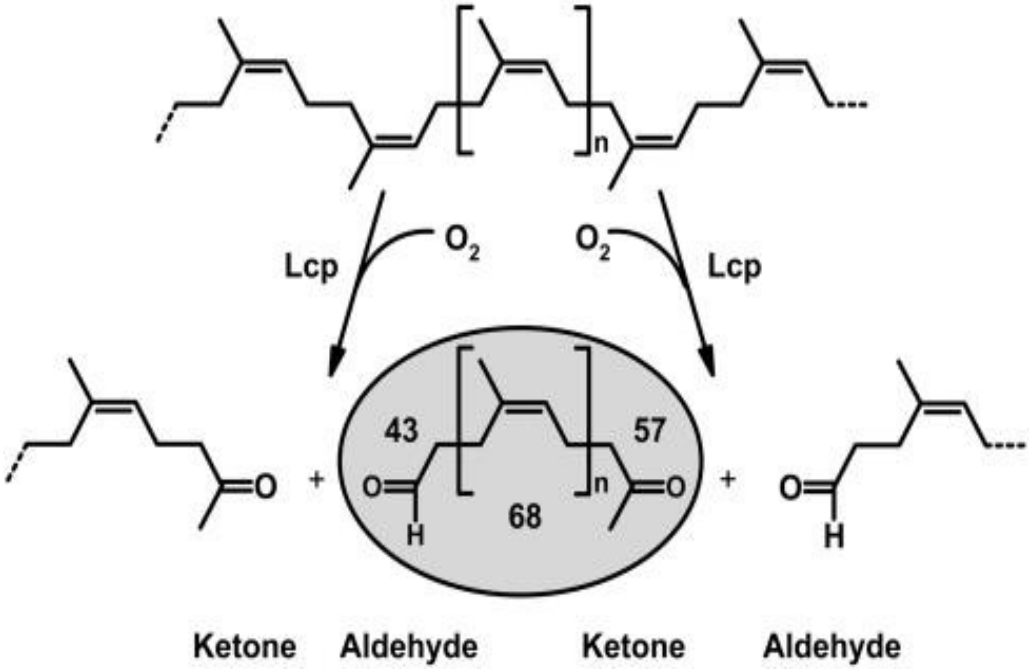
According to previous research, natural rubber degrading bacteria mostly belong to the group of Actinomycetes. Recently, certain thermophilic bacteria were also reported to be rubber degrading. Degradation of natural rubber latex by two gram negative bacteria, *Xanthomonas* sps. and *Pseudomonas aeruginosa* were reported in previous works. But there were no reports on gram positive bacteria other than the Actinomycetes. Biodegradation of natural rubber latex is a rare event.

With regard to their decomposition strategies, two different groups of rubber degrading bacteria can be distinguished. While bacteria forming clear zones (translucent halos) on latex containing mineral agar have been repeatedly described, only few representatives of the second, adhesive growing group were so far isolated and described and were classified into the so-called CMN group (*Corynebacterium, Mycobacterium, Nocardia*). Of these, *Gordonia polyisoprenivorans* Kd2 (DSM 44302T) is the most comprehensively characterized and taxonomically investigated strain. Until now only species belonging to the genera *Gordonia* (formerly known as *Gordona*), *Mycobacterium* and *Nocardia* were identified as non-clear zone forming rubber degrading bacteria that are dependent on direct contact to the substrate. Compared to clear zone forming rubber decomposing actinomycetes, the adhesively growing bacteria represent the more powerful rubber degrading bacteria.

THEORETICAL FRAMEWORK

The scientific basis is the depolymerization of poly(cis 1, 4-isoprene) of the equivalent enzymatic reaction. Tsuchi and co-workers were the first researchers to isolate and identify low-molecular-mass oligo(cis 1, 4-isoprene) derivatives with aldehyde and keto end groups from rubber-grown cultures of *Xanthomonas* and *Nocardia* species. Expression of *lcp* gene in *Streptomyces lividans* TK23 resulted in the accumulation of 12-kDa degradation products containing aldehyde groups, which are metabolized via beta-oxiditive pathway. Xanthomonas polyisoprene enzyme gene has the name Rubber oxygenase A (*roxA*), which revealed two heme binding motifs. In the *Nocardia*, and *Xanthomonas* the dioxygenase endocleavage of the double bond is the initial step.

Ibrahim et al., (2006) proposed a pathway for the cleavage of poly (cis-1,4-isoprene) via the *lcp* gene. Two individual cleaving reactions presumably catalyzed by Lcp result in the formation of a bifunctional isoprenoid species with a keto function and an aldehyde function.



The feasibility of using solid waste and effluent output from the natural rubber factory showing sizeable sources of valuable spent resources for bioconversion is shown in the figure below.

By-product/ Solid Waste/ Effluent (Based on Production Capacity 11,000 tons/yr)
Xuan Lap Natural Rubber Factory, Vietnam.

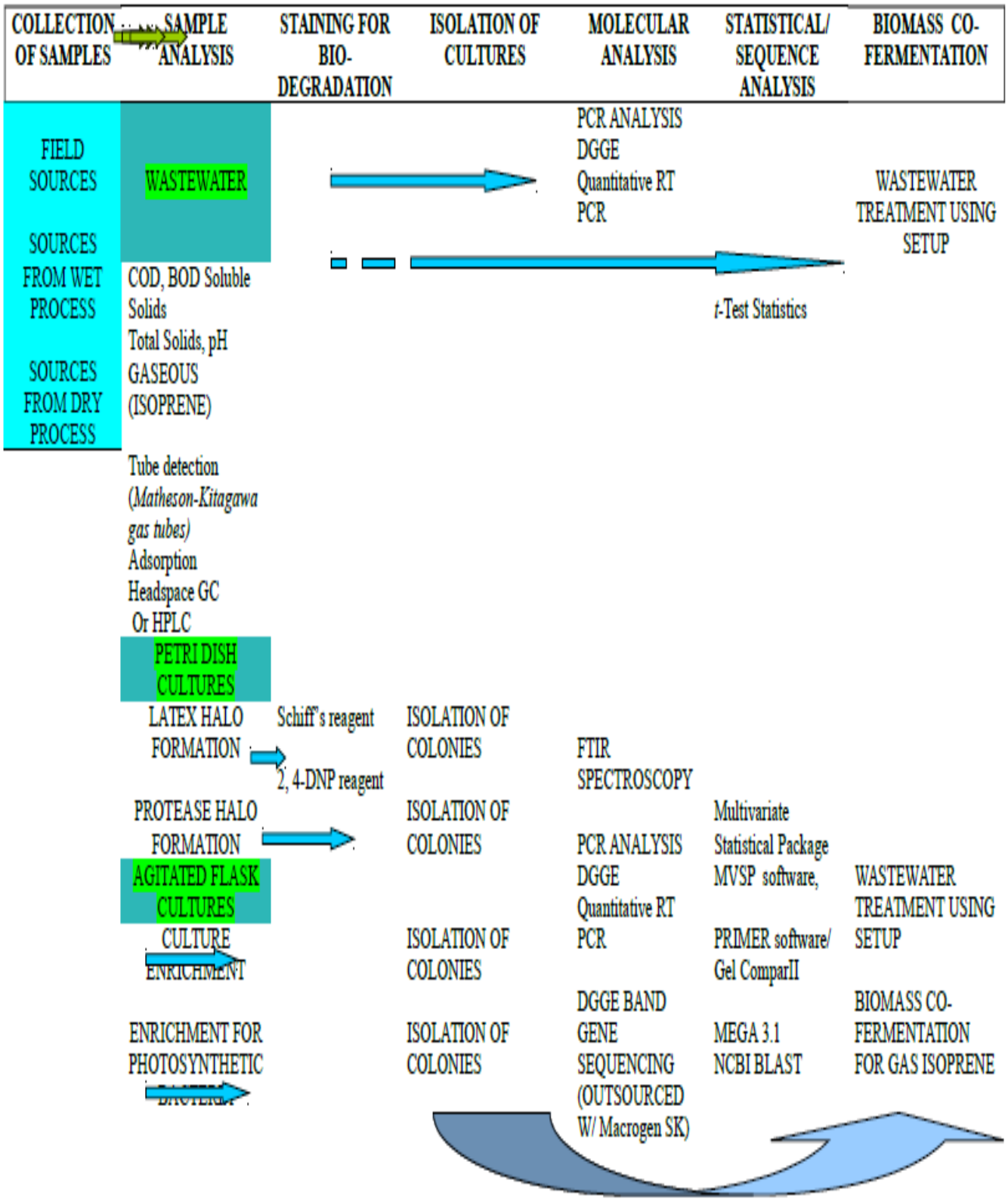
Inputs	**Kg/day**
Field Latex	106,000
Chemicals	476.2
Water	554,000
TOTAL	**660,476.2**

OVERALL CENTRIFUGATION PROCESS

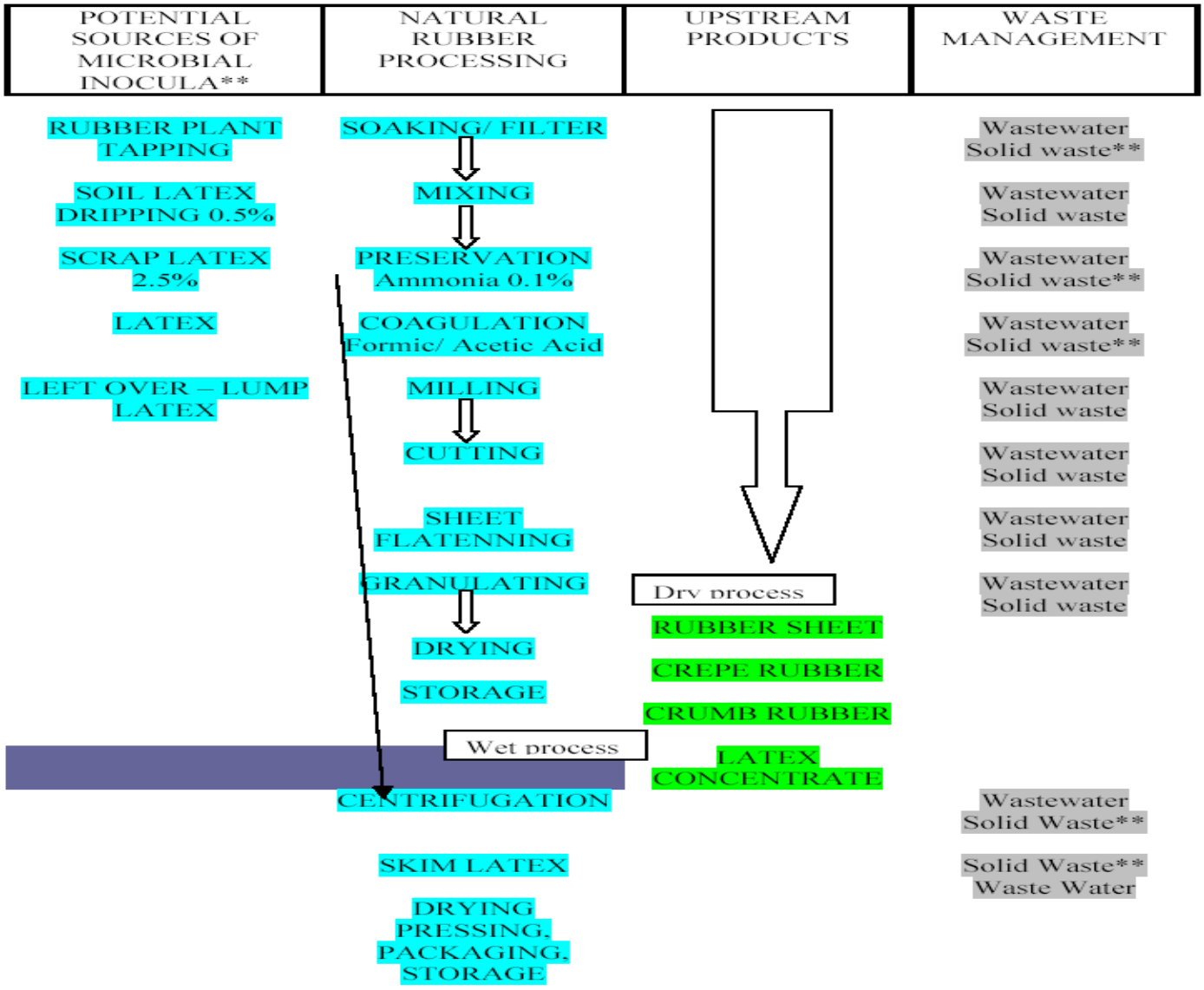
Output	**Kg/day**
Centrifuged latex	35,000
Skim latex	7,740
Gaseous	(Could be considered in the unaccounted 48,000kg/day lost in the output balance)*
Wastewater	570,000 (high Nitrogen, Ammonia)
TOTAL	**612,740**

METHODOLOGY

Experimental Design – (Please see separate next page.)



Sources of Microbial Inocula along the upstream processing of natural rubber are shown in the next flow diagram:



PREPARATION OF MATERIALS

Rubber latex materials. Rubber latex will be prepared from freshly tapped *Hevea brasiliensis*. crude latex contains approximately 35% rubber and 1 to 1.5% proteins.

Latex will be purified from soluble proteins by repeated (three times) centrifugation and washing with 0.002% Tween 80. The top layer (cream) from each centrifugation step is used for the next centrifugation step, while the bottom fractions are discarded. Latex is heat sterilized and stored at 4°C. Analytical grade rubber latex concentrate will also be purchased from local distributors.

Adsorbents for gaseous products isoprene to be used are matrix of Tenax® TA, Activated coconut charcoal sorbent tubes, Supelpak 2SV Matrix (purified Amberlite XAD-2 resins). Some reagents will be obtained commercially packed in tubes, Supelpak 2SV Matrix will packed in stainless steel tubes to be obtained commercially.

Bacteria, media, and culture conditions are to be made in nutrient broth or in a mineral salts medium described by Tsuchii and Takeda with 0.5% glucose or 0.2% purified rubber latex at 30°C. Latex cultures also contained 0.002% Tween 80 and sometimes contained 0.05% yeast extract. Solid media contained 1.5% agar. Latex agar will be prepared by the overlay technique; a bottom layer (~30 ml) of mineral salts agar in a petri dish overlaid with the same agar supplemented with 0.2% purified latex from *H. brasiliensis* (percentage of solid rubber) with or without 0.05% yeast extract, resulting in an opaque overlay. Colonies are screened for translucent clearing zones upon incubation at 30°C within 2 to 4 days, indicating utilization of the latex. Enrichment of photosynthetic bacteria such as *Rhodopsuedomonas* (an example of purple nonsulfur phosynthetic bacteria PNSB) will be done using high lux illumination light source during microbial isolation.

Wastewater pollution indicators tests for COD, BOD, TSS, SS, pH, temperature will be analyzed using standard methods. COD will be measured using the HACH Spectrophotometer; TSS, SS will be analyzed using pre-weighed filters after oven-drying.

Determination of Isoprene Gas from Sample/ Culture Preparation Gas formed in flask-incubated samples from various latex effluent or cultures will be trapped using a temperature-defined (for isoprene adsorption) collecting system in closed temperature vessel with adsorbent tube (packed commercially or laboratory prepared) attached to a vacuum/ aspirator/ pump. A semi quantitative method will use a Matheson-Kitagawa Toxic Gas Tube Detector System with a measuring range of 1 to 16 ppm, which is connected to an air sampling pump. Glass vessels are preferred in our experiments since plastics are permeable to light hydrocarbons and have been shown to absorb them. The gaseous hydrocarbon mixing ratios in the headspace (HS) above the medium are defined as ‘‘control’’. The emissions from samples are defined as positively occurring only when the HS mixing ratio for a given sample is higher than the control HS mixing ratio.

A 200 ml of sample suspension (collected from various latex process points) or sample cultures will be separately transferred from Nalgene incubation bottles into 250 ml Duran glass bottles fitted with a PTFE-septum or a suitable amber bottle filled before analysis. A further identical glass bottle (volume) is left empty as a gas blank and one was filled with the same liquid concentration of blank medium. A head space gas chromatograph/mass spectrometer (HS-GC/MS) instrument will be used for the analysis of VOCs in the headspace of the samples. A volume of 10 mL of headspace sample will be cryogenically concentrated at -70 oC (with a circulation cooler) in a stainless steel microtrap packed with porous silica beads (Unibeads 1S, 80/100 Mesh, Alltech) under a flow-rate of 40 mL min-1. A RTX-VMS capillary column (40 m-long, 0.18 mm ID, 1 mm film thickness) supplied by J & W Scientific (California, USA) or a suitable column will used for the separation of sampled compounds. After sample injection, the column oven will be maintained at 50 oC for 4 min. After the initial isothermal step, the temperature is first increased to 100 oC at 9 oC min-1 and then from 100 to 230 oC (2 min) at a rate

of 40 oC min-1. The mass spectrometer detector will be operated in electron impact mode with the following conditions: potential ionization 70 eV; source temperature 230 oC ; and selected ion monitoring (SIM) mode. (The detection limit is in the range of 0.05 to 5 pptv and the uncertainty of 15%.) For each at least 3 replicates will be collected and analysed.

Extra-cellular protease activity is assayed with azocasein (0.1 mg/ml) as substrate in a reaction mixture of 1 ml of Tris-HCl 0.1 M, pH 9, 200 ml of CaCl2 0.2 M and 50 ml of azocasein, and 1 ml of sample supernatant and further incubated at 37 °C for 1 hr. After which, trichloroacetic acid 5% (1.5 ml) was added. The proteolytic activity is defined as the change of one absorbance unit at 440 nm. Isolates are screened for extra-cellular proteolytic activity in culture petri dishes containing gelatin/ or casein.

Detection of aldehyde groups with 2, 4-DNP reagent. 1 mL of 2, 4-DNP reagent will be added to the sample, and the yellow precipitate that developed over 1-2 min at room temperature is noted. Any precipitating yellow colour denoted the presence of aldehyde groups produced during the degradation of the polymers. The composition of the 2, 4-DNP reagent is as follows: 3 g of 2, 4-dinitrophenyl hydrazine dissolved in 15 mL of sulfuric acid plus 70 mL of 95% ethanol plus 20 mL of H2O. Aldehyde group staining of sample preparations will also be compared with the adsorbed gaseous isoprene formation.

DNA extraction from microbial cultures will use the Fast DNA Spin Kit (MPbio.com) and will be performed according to kit protocols. DNA extraction from wastewater samples will be using the PowerWater® Sterivex™ DNA Isolation Kit (Mo-Bio.com) Extracted DNA samples obtained by the two methods will be purified using a PCR purification kit, and DNA concentration is measured using an UV/Vis spectrophotometer, at 260 nm wavelength. Coefficient at 260 nm (i.e. an A260 of 1 gives the following µg/ml): DNA:

50; RNA: 40; Oligos: 33. The DNA extracted is resuspended with 100 ul of sterile distilled water and stored at -20 C until use.

Primers

The 17-mer canonical forward primer, designated F-968 (Brons and van Elsas 2008) amplified the 14 different bacterial phyla namely, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Chloroflexi*, *Gemmatimonadetes*, *Chlorobi*, *Bacteroidetes*, *Cyanobacteria*, *Chlamydiae*, *Ferribacter*, *Deinococcus*, and candidate division TM7, the dominant phyla being *Firmicutes* and *Proteobacteria*. The bacterial 16S rRNA specific primer F-968 (5′-AA CGC GAA GAA CCT TAC-3′), to which a 40-mer GC clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G) is attached at the 5′ end. The reverse primer R1401-1b CGG TGT GTA CAA GAC CCG GGA ACG is specific at about position 1400.

lcp gene primers are as follows:

forward primer 5'-ATGGAGAATCTCAGCAGGCGA

reverse primer 3'-GGTCAGCCCGGCCTGTTG.

roxA primers are as follows:

(i) P1-R2: 5P-AARTCRTGSCCSCCRTTRTC,

(ii)P1-NR2A:5P-TCRTGSCCSCCRTTRTCRTTRCC,

(iii) P3-F5: 5PTGGGGSCTSCCSAACWSSGCSAACGAYGC,

(iv) P3-NF5: 5P-CCSAACWSSGCSAACGAYGCNGG.

expected PCR product 1.3 kbp

primers for α-methylacyl CoA racemases

5´-GGA TCC AGG GAG GAC GTC CAT GAC AGC AGA TTC GAC AC

3´-TCT AGA TCA GTC GGT CCA GAT GGT G

Primers will likewise be generated and selected from the following Open Reading Frame (ORF) genes specified for the corresponding enzymes involved in the beta-oxidation pathway for rubber latex biodegradation (as described by Hiessl S et al. 2012) using the Open Reading Frame Retrieval java tool.

Acyl-CoA synthetase

GPOL_c26980

GPOL_c49330

Acyl-CoA dehydrogenase

GPOL_c06060

GPOL_c10630

GPOL_c11980

GPOL_c15560

GPOL_c36890

GPOL_c45280

GPOL_c45460

2,4-Dienoyl-CoA reductase

GPOL_c19120

Enoyl-CoA hydratase/isomerase

GPOL_c09320
GPOL_c30630
GPOL_c36880
GPOL_c41700
GPOL_174p01070

3-Hydroxyacyl-CoA dehydrogenase
GPOL_c09390

Thiolase
GPOL_c05990
GPOL_c14950
GPOL_c18410

α-Methylacyl-CoA racemase
GPOL_c36450

Ribosomal Database Collection II Release 9.50 (option Probe Match; http://rdp.cme.msu.edu/probematch/search.jsp) will used to analyze the 16S rRNA gene sequences.

PCR amplification.

PCR mixtures are composed as follows. Seven microliters of 10× PCR buffer 100 nmol $MgCl_2$, 0.5 µl formamide, 0.5 µg T4 gene 32 protein, 10 nmol of each deoxyribonucleoside triphosphate, 10 pmol of each primer, and 3 U of 10 U/µl Ampli *Taq* DNA polymerase, Stoffel fragment (Applera), are combined with st.H_2O to 50 µl in a 0.2-ml Microfuge tube. After the addition of 5 ng of template DNA, the mixtures are incubated in a PCR system programmed as follows: initial denaturation of double-stranded DNA for 5 min at 94°C; 10 (touchdown) cycles consisting of 1 min at 94°C, 1

min at 60°C, and 2 min at 72°C with a decrease in the annealing temperature of 0.5°C per cycle; 25 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; and extension for 30 min at 72°C. All amplification products were purified with the Wizard PCR DNA purification system (Promega, Madison, WI) and analyzed by electrophoresis in 1.0% (wt/vol) agarose gels, followed by ethidium bromide staining (1.2 mg/liter ethidium bromide in 1× Tris-acetate-EDTA).

Nested PCR DGGE

Three-step nested-PCR-DGGE.Two strategies will be used to analyze the bacterial communities. First, the 16S rRNA fragment will amplified using the primer pair F-968(GC)/ R1401-1b. The PCR was performed using a touchdown annealing protocol with decreasing timperature. Second, a three-step nested amplification was performed to obtain different rubber biodegradation primers (*lcp, roxA, mcr*) associated 16S rRNA fragments suitable for DGGE. In the first step, a nearly complete 16S rRNA gene fragment will be amplified using a canonical primer pair F-968 . The product obtained will used as a template for a second amplification with rubber group-specific primers. Finally, to generate products suitable for DGGE, a third round of amplification will be performed with DGGE primers GC clamped using the product of the second round as template.

A total of 300-500 ng of PCR product is loaded into each lane for wastewater community DGGE, while separately, 50 ng of DNA is loaded for pure-culture DGGE. A denaturing gradient of 35–65% denaturants [100% denaturants is a mixture of 3.5 M urea and 32% (v/v) formamide] is used in 6% (w/v) polyacrylamide gels. Electrophoresis was performed in 0.5 × Tris-acetate EDTA buffer at 60 °C and at a constant voltage of 70 V for 16 h using a DCode system (BioRad). The wells are loaded with roughly equal amounts of DNA, and electrophoresis is carried out in 0.5× TAE buffer at 70 V for 16 h

at 60°C. The gels were stained for 90 min in 0.5× TAE buffer with SYBR gold (final concentration, 0.5 µg/liter; Invitrogen) or an alternative staining. Gel images will be captured using a Gel Doc (BioRad), and analyzed using quantity one software (BioRad). For this analysis, each DGGE band is assumed as operational taxonomic unit (OTU) or phylotype. Bands will be detected using the band-searching algorithm of the software, which takes care of background subtraction. Gels will be checked visually for ensuring the number of bands. The background is subtracted using a rolling disk set at 20, and band density at positions is converted to intensity per *R*f value between 0 and 1. After normalizing for total intensity across lanes, data were input into the past software package and analyzed using multivariate principal component analysis (M Statistical Package (MVSP v3.1) software, and PRIMER X software for non-parametric multidimensional scaling (NMDS). Alternatively, the DGGE patterns will be compared by clustering the different lanes by Pearson's product-moment correlation coefficient with GelCompar II software (Applied Maths) by the unweighted-pair group method with arithmetic mean, rolling-disk background subtraction, and no optimization.

Quantification of functional genes

RNA from microbial cultures will be isolated using Qiagen RNeasy Mini kit. Alternatively extracted DNA will be used for qRT PCR. qRT PCR will be used to quantify the abundance of *lpc, roxA, mcr* and selected genes of the beta-oxidation pathway in rubber bioprocessing. All reactions will be conducted on a BioRad real-time PCR instrument in 25-µl reaction mixture volumes. The PCR chemistry was based on QuantiTect Sybr green *Taq* and buffer (Qiagen). The quantification of the *lpc, roxA, mcr* and selected ORFs of the beta-oxidation pathway are based on the given primer sequences provided above. Primers are added to give 0.4 to 0.8 µM in the PCR master mix, and 5 µl of DNA is pipette into each reaction. The themocycle conditions are based on touch-down PCR. In the first 8 cycles, the annealing temperature is

decreased from 60°C to 55°C, and then maintained at 55°C for a further 30 cycles. Denaturation is at 94°C for 30 s, primer annealing for 30 s, and extension at 72°C for 45 s.

Outsourcing of sequence analysis, construction of trees, and statistical analyses

To obtain a substantially pure PCR product for DNA sequencing, individual bands from DGGE gels are carefully excised using sterile razor blades, placed in 1.5-ml microcentrifuge tubes containing 40 µl of 1× Tris-HCl buffer, and stored for 48 h at 4°C. Analysis of the sequences will be done with Macrogen, South Korea. Chimera check with Bellerophon was used to check for chimeric sequences (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi). Bellerophon is a program for detecting chimeric sequences in a multiple-sequence data set by comparative analysis. It is specifically developed to detect 16S rRNA gene chimeras in PCR clone libraries but can be applied to other gene data sets. The partial 16S rRNA gene sequences are compared with sequences in GenBank with nucleotide-nucleotide BLAST (BLAST-N) to obtain the nearest phylogenetic neighbors (www.ncbi.nlm.nih.gov/BLAST/). Sequences showing more than 97% similarity are considered to belong to the same operational taxonomic unit (OTU). Trees are constructed from libraries obtained with each reverse primer by neighbor joining within the program MEGA 3.1 (The Biodesign Institute) and bootstrapped with 500 repetitions. These trees are used to obtain broader groupings—supported by checks with the Ribosomal Database Project (RDP) database—which will served to prepare histograms revealing the compositions of the bacterial communities.

FTIR-ATR spectroscopy Sample aliquot will be subjected to FTIR Spectroscopy. Spectra will be recorded by a Fourier transform infrared (FTIR) spectrometer (Thermo Scientific Instruments) with the attenuated total reflectance (ATR) technique. The angle of incidence is set at 45° by using a ZnSe crystal with 20 active internal reflections. Sixty

scans will be co-added with a resolution set at 4 cm^{-1}. Transmittance will be recorded using Thermo Scientific software with baseline analysis. For comparative analysis, spectra are standardized by applying a vector normalization. No further spectral processing will used to ensure band frequency and band shape quality. For spectral control, measurements in the transmission mode had been performed by using ZnSe disks as sample holders.

Bench-scale fermentation will be performed using wastes from primary processing of rubber. Microbial isolates biomass obtained would be co-fermented with the rubber waste using a suitable preliminary flask setup. Test conditions will be designed to process the waste as well as to be able to recover as much the gaseous by product A suitable material interface will be used in the initial set up for entrapment and adsorption of gaseous products and commercially available activated carbon and hybrid products available. A bench-scale bioprocessing setup will also be designed for the treatment of latex waste effluent using microbial biomass. Estimates for floc-granule formation will be based using a test procedure in a beaker reaction to determine the conditions and residual pollution indicators.

General Statistical Analysis Descriptive statistics include the mean and standard deviation or the median and 5th and 95th percentiles, where specified. P-values <0.05 were considered statistically significant. The *t*-Test based on F values will be used to analyze the difference between treated and wastestream COD/ BOD values. A Mann-Whitney-U-test will be used to test differences in peak area per compound between treated and control sample preparation. A non-parametric Spearman's rank correlation coefficient will be assessed between the culture and qPCR measurements to compare quantitative results.

EXPECTED OUTPUT

Microbial strains will be characterized and possible properties for isoprene processing as well as extra-cellular protease activity. A bench-scale microbial bioprocessing setup will be designed for the recycling and treatment of wastes from the primary processing of rubber. Initial studies will include polymer entrapment of volatiles to measure production of isoprene.

TARGET BENEFICIARIES

- Natural rubber latex tappers
- Natural rubber and technically specified rubber users and producers

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CHAPTER 4

LACCASE PRODUCTION USING LOCALLY CULTIVATED MUSHROOM

NOEL M. UNCIANO, EMILIO MONTIAGUE, JOHN PAULO JOSE

& URSELA G. BIGOL

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ABSTRACT

Highly active Laccase enzymes were produced by mushroom cultures of Oyster and *Ganoderma lucidum* at a relatively short time gap of less than a month. Solid cultures produced more Laccase with a peak activity of more than 21K U/ml for Oyster using wheat. Oyster cultures were more active with agricultural substrates: corn, sorghum, sweet potato and wheat; while *G. lucidum* cultures were more active with sorghum, sweet potato and wheat. High specific activity of more than 700 U/mg was observed with sorghum and slightly higher with wheat at 756 U/mg. A change in activity of 445x was observed at week 3 for Oyster solid cultures. Ferrous ions inhibited laccase at 99.7%; Mn and Cu at 10%; and Ca at 6%. Li ions and 2,4 D moderately increased Laccase at more than 16%; Al at 10%; and Zn or diphenylamine at 8%. Partial purification of Laccase with ammonium sulfate resulted in more than 3x fold increase in activity. Enzyme product recovery with course filtration was quite high at more than 99%. Data model analyses showed that for Corn and Wheat, the yield of laccase specific enzyme activity was best described by a model similar to the Lineweaver-Burk, while Sweet Potato was described by 2 models: a) linear and b) square root-Y logarithmic-X model. The latter was the only model, which have estimated significant intercept and slope values. Sorghum substrate did not produce a comparatively significant effect on the yield of laccase during the four-week time course experiment.

THE EMERGENCE OF THE BIOECONOMY

The twenty-first century has been characterized by the emergence of new challenges faced by globalization amidst the need for new socioeconomic and resource scarcity caught by rapid urbanization and population surge, environmental protection and regulation, an expanding global class hungry for automobiles and modern technology, and more volatile finances that face the global market. The term Bioeconomy was the product of these global mosaic of challenges. What Golden & Handfield (2014) had put it, Bioeconomy is global industrial transition of sustainably utilizing renewable aquatic and terrestrial resources in energy, intermediate, and final products for economic, environmental, social, and national security benefits. The White House (2012), declared "bioeconomy is one based on the use of research and innovation in the biological sciences to create economic activity and public benefit." Quoted in the Organization for Economic Co-operation and Development (OECD 2009): "From a broad economic perspective, the bioeconomy refers to the set of economic activities relating to the invention, development, production and use of biological products and processes. If it continues on course, the bioeconomy could make major socioeconomic contributions in OECD and non-OECD countries. These benefits are expected to improve health outcomes, boost the productivity of agriculture and industrial processes, and enhance environmental sustainability."

OBJECTIVES

To isolate and screen mushroom species for laccase production.

To produce laccase by submerged fermentation and solid cultures..

To optimize the fermentation parameters

SIGNIFICANCE

In dried wood and trees are several mushroom species that are potential sources of laccase. Commercial mushrooms are alternative sources should collection from the forest will not be possible. These can be cultured through submerged fermentation and the broth will be processed to determine the yield of production, isolate the enzyme and could potentially be applied to various industrial processes.

Laccase enzymes have gained industrial application in the field of textile dye decolourization, delignification of pulp, and paper effluent detoxification, and food industry. Recently, these are used in the design of biosensors, biofuel cells, as a medical diagnostics tool and bioremediation agent to clean up herbicides, pesticides and certain explosives in soil; as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics.

LITERATURE

Laccases (Phenoloxidases, EC 1.10.3.2) are outstanding because they use molecular oxygen, which is the final electron acceptor (in multicopper reactive sites) as a co-substrate instead of hydrogen peroxide as used by peroxidases. These are widely found in plants and fungi, in some bacteria and insects, and oxidizes polyphenols, methoxy substituted phenols, aromatic diamines and a range of other compounds.

These enzymes have received attention of researchers in the last few decades due to their ability to oxidize both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants with the use of “mediators.” Laccases

are increasingly being used in food industry for production of cost-effective and healthy foods. Their baking applications are due the ablity to cross-link the esterified ferulic acid on the arabinoxylan fraction of dough, resulting in a strong arabinoxylan network. It was also reported that laccase may improve crumb structure and softness of baked products increases in strength and stability, as well as reduced stickness of dough, which confers improvement of baking properties. The advantages of laccase compared to other enzymes include relatively high yields, uncomplicated isolation from bulk fungal cultures, easy screening for specific producers with a broad substrate spectrum, a high degree of stability and activity especially after immobilization; broad specificity (which allows them to transform a wide range of substrates) and to their wide diversity, most fungal laccases are very stable, especially at pH near neutrality (glycosylation seems to be implicated in the stability of fungal laccases), their organic substrate oxidation site exhibits a high redox potential (around 0.78V/normal hydrogen electrode (NHE)) and, finally they use dioxygen, a harmless and abundant compound, as a co-substrate instead of peroxide as other oxidases such as peroxidases.

A number of studies have confirmed the potential of laccase-mediator systems for paper pulp delignification, pitch control, polymer modification, other applications in the forest industry; and bioethanol production from physically and/or chemically pretreated lignocellulose. However, most of the studied mediators are synthetic compounds based on nitrogen heterocycles whose high cost and potential toxicity make it difficult to implement at an industrial scale. Some natural phenols, which form stable aromatic radicals, are being studied as mediators for bio-bleaching and removal of lipophilic extractives from paper pulp.

The use of excessive concentrations of glucose and sucrose as carbon source in cultivation of laccase producing fungal strains has an inhibitory effect on laccase production. This could be improved by using polymeric substrates like cellulose as carbon source during cultivation. At 1% (w/v) carbon sources such as: glucose, mannose, maltose, sucrose, fructose, cellobiose, cellulose, glycerol and lactose are the

commonly used carbon sources. Fructose was shown to be a good carbon source for laccase production in *Pleurotus sajor-caju*, cellobiose in *T. pubescens*, and lactose or glycerol in *Pseudotrametes gibbosa*, *Coriolus versicolor* and *Fomes fomentarius*. Yeast extract, peptone, urea, (NH4)2SO4, and NaNO3 are the commonly used nitrogen sources. Studies show that the elevated laccase activity was achieved by using low carbon-to-nitrogen ratio while others show that it was achieved at high carbon-to-nitrogen ratio. Agricultural residues can be used as carbon and nitrogen sources for laccase production.

Laccases were generally produced in low concentrations by laccase producing fungi, but higher concentrations were obtainable with the addition of various supplements to media. The addition of aromatic compounds such as 2,5-xylidine, lignin, and veratryl alcohol is known to increase and induce laccase activity. Many of these compounds resemble lignin molecules or other phenolic chemicals. These compounds affect the metabolism or growth rate while others, such as ethanol, indirectly trigger laccase production. Soya oil as inducer of laccase activities, has attained 4- fold higher than those obtained in the reference cultures. The addition of 150 μM copper sulphate to the cultivation media can result in a fifty-fold increase in laccase activity compared to a basal medium. Ferulic acid or vanillin proved to increase the laccase production up to 10 times in *Pleurotus pulmonarius*. Vitamins like biotin, riboflavin and pyridoxine hydrochloride as well as amino acids such as methionine, tryptophan, glycine and valine stimulated laccase production in *Cyathus bulleri*, whereas cysteine inhibited the production. Antibiotics like apramycin sulfate stimulated laccaseproduction in *Cyathus bulleri* and *Pycnoporus cinnabarinus*. Metals like Mn2+ led to a 4.5-fold increase in the laccase productionby *Coprinus* comatus. Laccase isozyme production is increased by 4.4 fold with 3.0 mM caffeic acid.

Most reports indicate that an initial pH of 4.5-6.0 and a temperature between 25-30 °C are suitable for laccase production. The effect of aeration varies between species; growth of some fungi is highly favored with aeration, while others can suffer from stress

caused by oxygen. In addition, as aeration can involve mechanical stirring, this may cause stress on the cells by rupturing them. Agitation is another factor which affects laccase production. Mycelia are damaged when fungus is grown in the stirred tank reactor and laccase production considerably decreased. Conversely, in some species, agitation did not play any role in the production of laccase by *T. versicolor*.

METHODOLOGY

Mushroom cultures were taken on stock in the laboratory (Culture Collection), or isolates taken from the wild or from cultures from RTU. The agricultural substrates used were corn, sorghum, sweet corn, and wheat at 2% media with dextrose. Growth fermentations used were solid culture fermentation in flasks and submerged fermentation in bioreactor flasks. Crude enzyme was harvested with filtration. Laccase activity was determined using ABTS as substrate (0.05 mM) in 100 mM Acetate pH 5.0. Various chemical additives (Fe, Cu, Mn, Ca, Zn, Li, 2,4 D, diphenylamine etc.) were incorporated at a final conc. of 0.2mM during the assay for enzyme activity effect determination. Enzyme product recovery after ammonium sulfate precipitation was done via coarse filtration instead of using centrifugation.

RESULTS AND DISCUSSION

We started with culture isolates grown in the laboratory, isolates taken from the wild and cultures from RTU, namely: Ganoderma lucidum (RTU), Ganoderma sp. (wild), Pleurotus florida (Angel mushroom=Oyster), Pleurotus cystidiosus (Abalone), and Lentinus edodes. Two mushroom cultures (Ganoderma lucidum (RTU), and Pleurotus florida (Angel mushroom=Oyster) were grown in simple media containing 2% agricultural substrate with dextrose and cultivated for growth optimization using submerged and solid substrate fermentations. Ganoderma cultured in submerged

fermentation showed substantial activities for production of laccase for substrates: sorghum, sweet potato and wheat. As shown in Fig.1, activities peaked with sweet potato after 3 weeks. (Please note that specific activity is mislabeled for U/ml in the figures 1 to 4.) Very high activities were obtained with Ganoderma cultures in solid substrates, sorghum and wheat. Peak activities of more than 4372 U/ml for sorghum and 1358 U/ml for wheat were observed after 3 weeks (Fig.2). Oyster cultures in submerged fermentation produced laccase actively after 3 weeks for substrates corn, sorghum and sweet potato with peak activity of 549 U/ml (Fig. 3). Oyster grown in solid cultures produced laccase at highest activities for week 3 at more than 21K U/ml using wheat; sorghum produced 10,700 U/ml (Fig. 4). Specific activities for these substrates are more than 700 U/mg and higher with wheat at 756 U/mg. Data were summarized in tables 1 to 4 for week 1 to week 4 data. Maximum change in activity, was at 445x for week 3 in solid substrate fermentation for Oyster cultures.

Using two-way ANOVA, significant differences were observed for submerged and solid fermentation (Fig 6).

Laccase activity was affected by different chemicals tested as shown in Fig. 5. Ferrous ions practically inhibited laccase at 99.7%; Manganese and copper at 10 %; and calcium at 6 %. Laccase activity was increased markedly with Lithium ions and 2,4 D at more than 16%; aluminum at 10%; and with zinc or diphenylamine at 8%.

Partial purification of laccase with ammonium sulfate resulted in more than 3 fold increase in specific activity at several concentrations: 50%, 70%, and 80% saturation.

Laccase enzyme yield based on the substrates corn and wheat (in solid substrate fermentation with Oyster mushroom) showed an interaction best described by a double reciprocal model (similar to the Lineweaver-Burk), while sweet potato was best described by linear and square root-Y logarithmic-X models (Figure 9). However, only the square root-Y logarithmic-X model for sweet potato was significant for both the

estimated intercept and slope values. Sorghum as used in our experiments did not produce a significant effect on the yield of laccase enzyme specific activity during four-week time course. Table 5, showed comparison of best-fit models for solid substrate fermentation. Figure 10 showed surface plots for wheat substrate (Fig. 10 A&B) and for corn substrate interactions (Fig. 10 C&D).

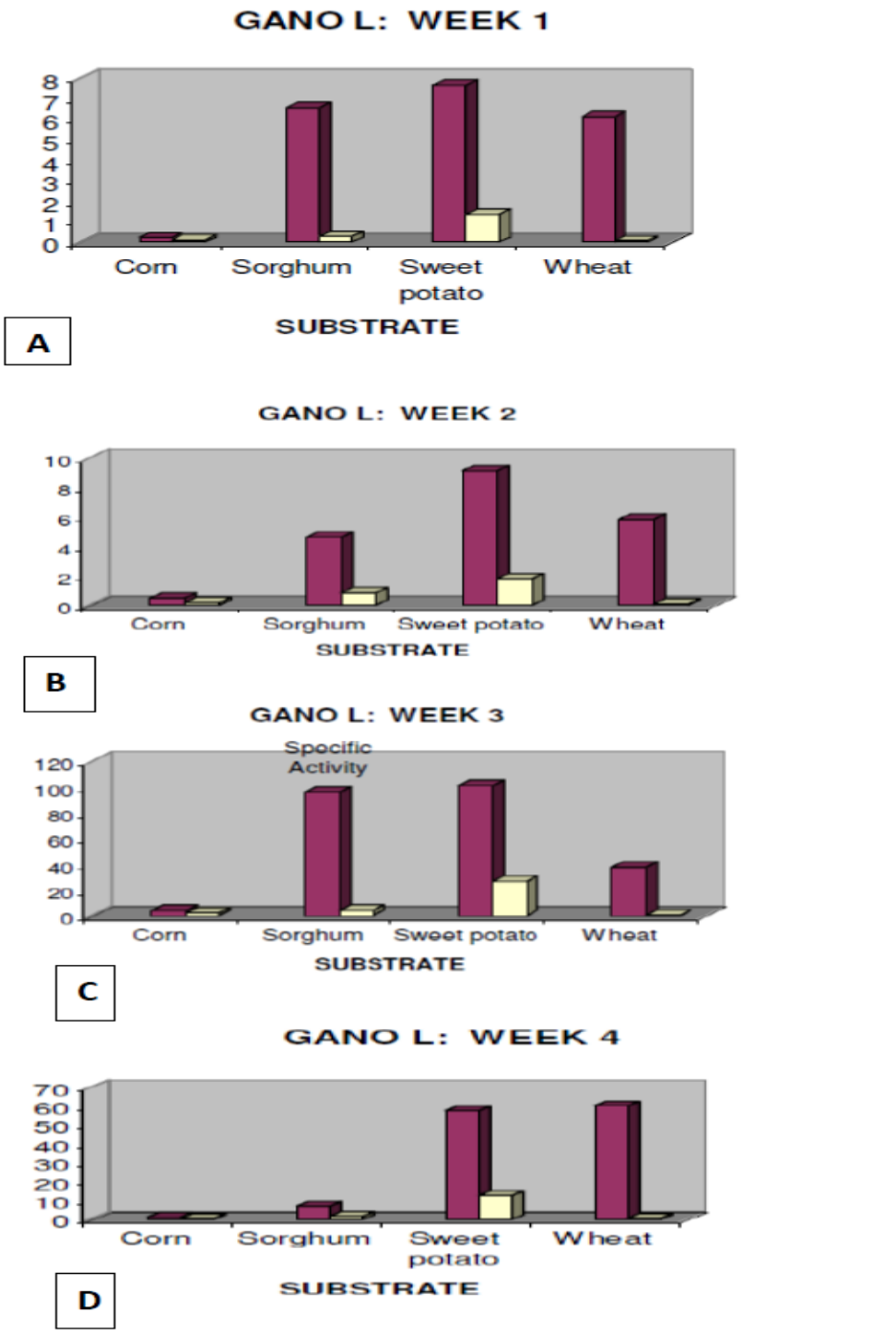


Figure 1. Ganoderma submerged fermentation

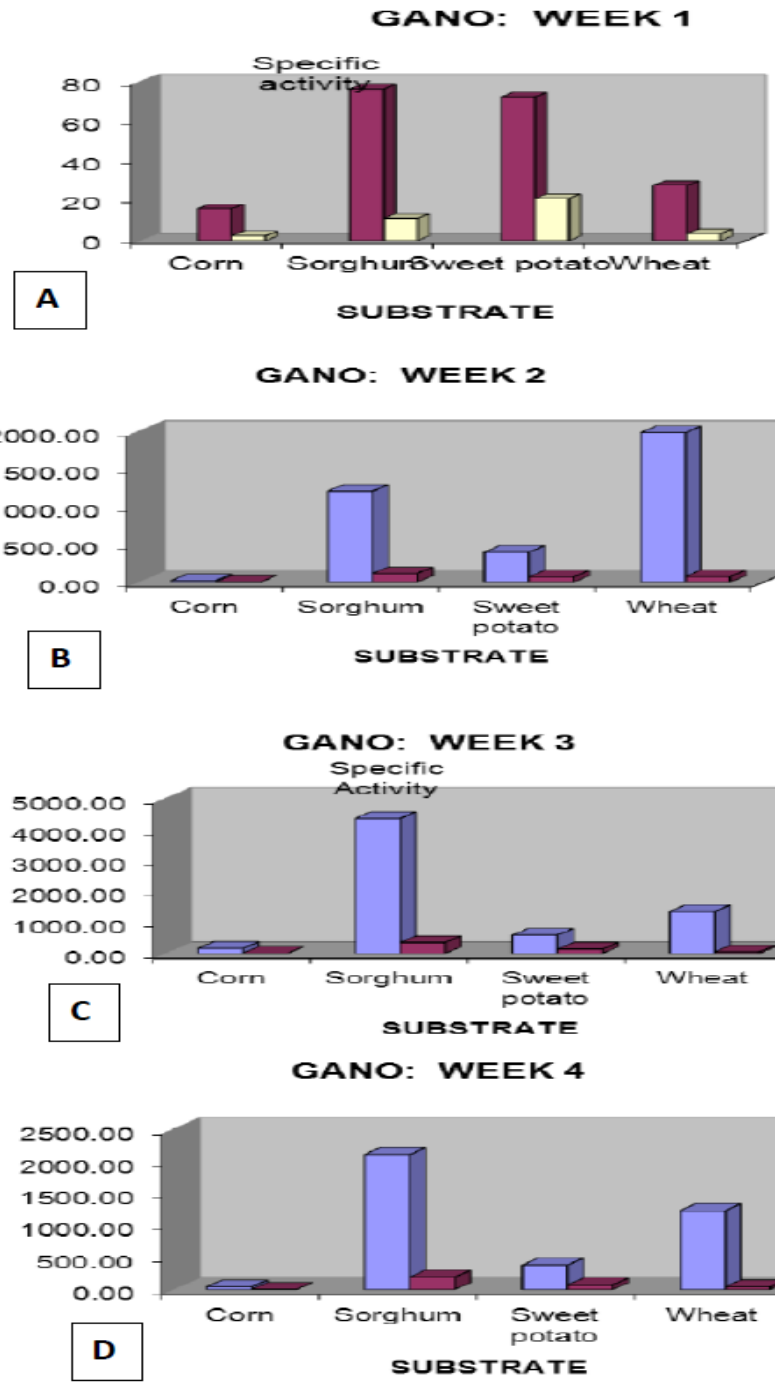
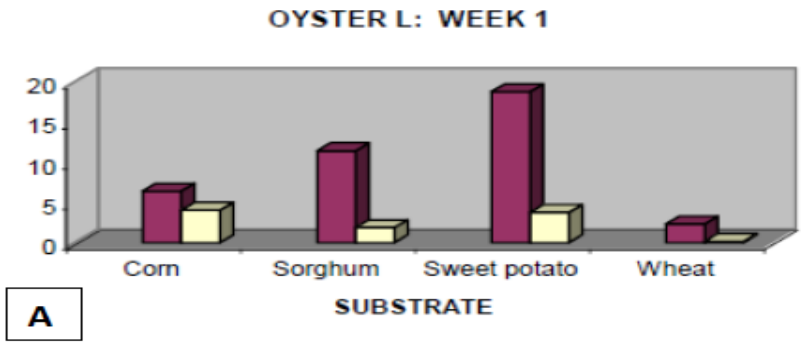
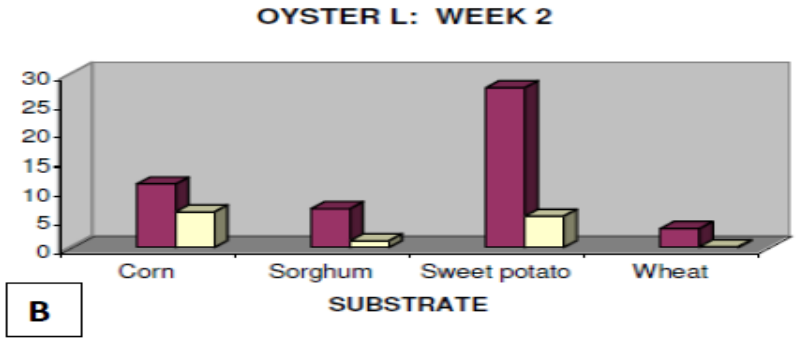


Figure 2. Ganoderma solid substrate fermentation



A



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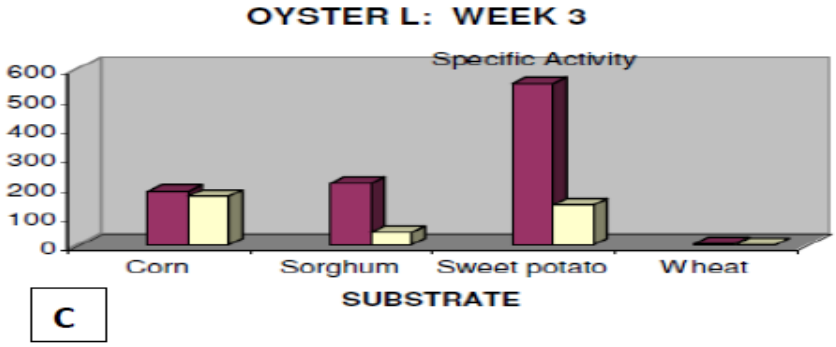

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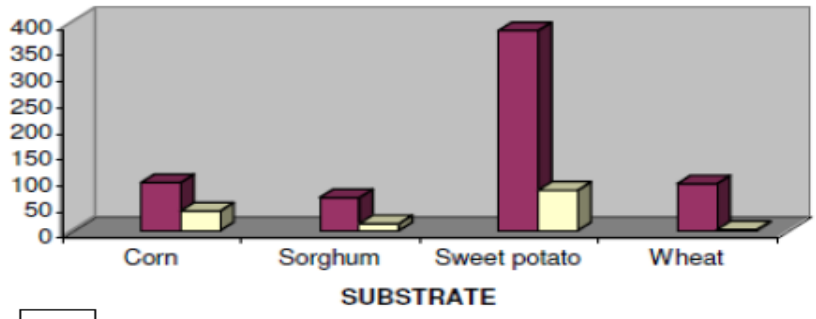

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Figure 3. Oyster submerged fermentation

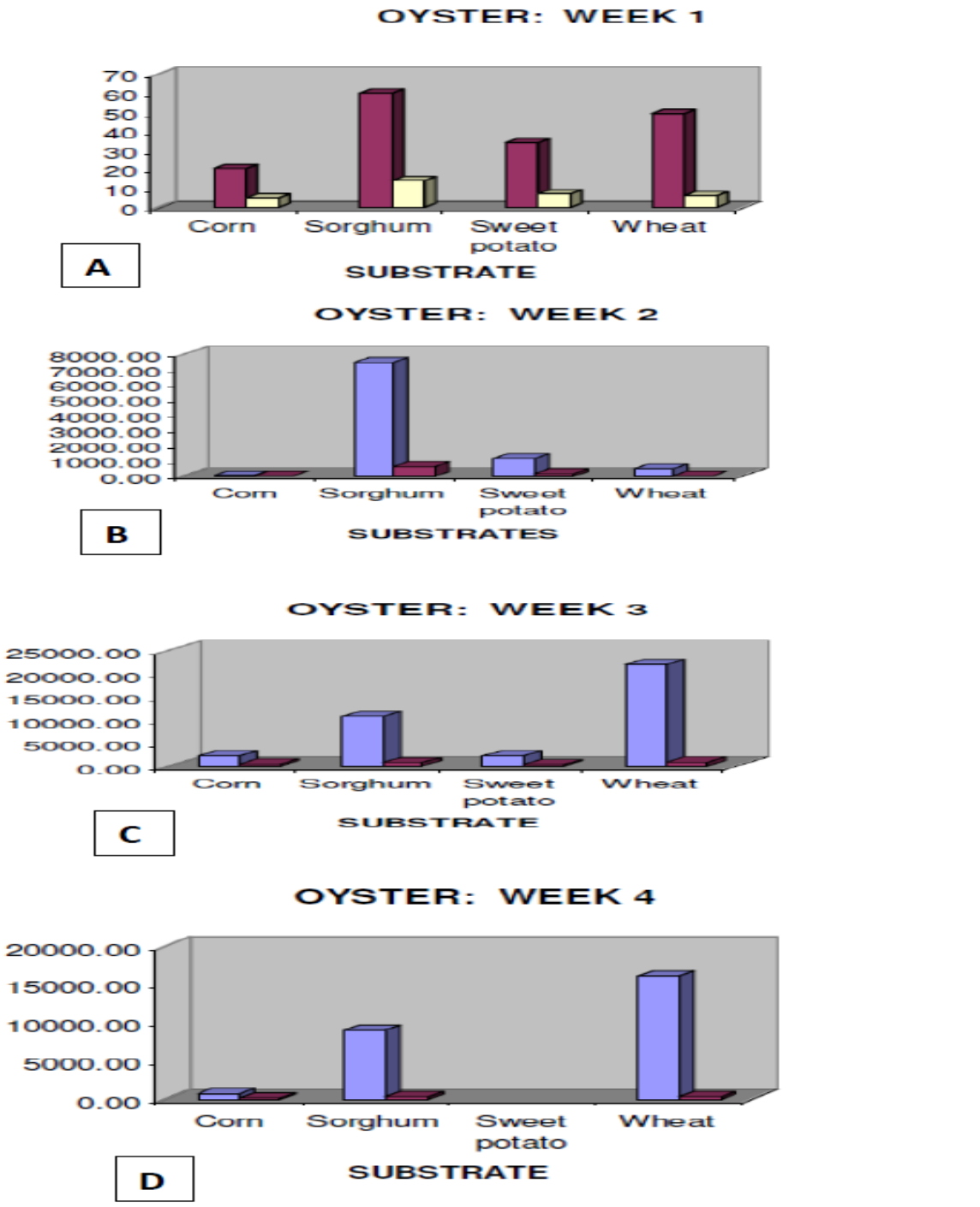


Figure 4. Oyster solid substrate fermentation

TIME-COURSE OF LACCASE FERMENTATION (WEEK 1 TO WEEK 4)

WEEK 1	U/mL	Specific Activity
SOLID FERMENTATION: OYSTER		
Corn	20.57	4.98063
Sorghum	60.13	14.14824
Sweet potato	34.04	7.19662
Wheat	49.35	6.19975
GANODERMA		
Corn	16.17	2.45745
Sorghum	76.58	11.26176
Sweet potato	72.47	21.31471
Wheat	27.86	3.54904
SUBMERGED FERMENTATION		
OYSTER		
SUBSTRATE		
Corn	6.39	4.01887
Sorghum	11.40	1.88430
Sweet potato	18.73	3.74600

Wheat	2.31	0.14574
GANODERMA		
Corn	0.23	0.11058
Sorghum	6.55	0.28416
Sweet potato	7.69	1.35149
Wheat	6.12	0.07823

Table 1. Data of Laccase activity after week 1 of fermentation.

WEEK 2	U/mL	Specific Activity
SOLID FERMENTATION: OYSTER		
Corn	56.76	9.60406
Sorghum	7445.56	617.37645
Sweet potato	1148.54	152.52855
Wheat	483.26	14.60000
GANODERMA		

Corn	20.49	1.25783
Sorghum	1210.21	115.36797
Sweet potato	399.03	70.50000
Wheat	1988.98	73.58417
SUBMERGED FERMENTATION		
OYSTER		
SUBSTRATE		
Corn	11.07	6.21910
Sorghum	6.79	1.18499
Sweet potato	27.66	5.46640
Wheat	3.31	0.14498
GANODERMA		
Corn	0.48	0.16609
Sorghum	4.62	0.84000
Sweet potato	9.14	1.79568
Wheat	5.84	0.07316

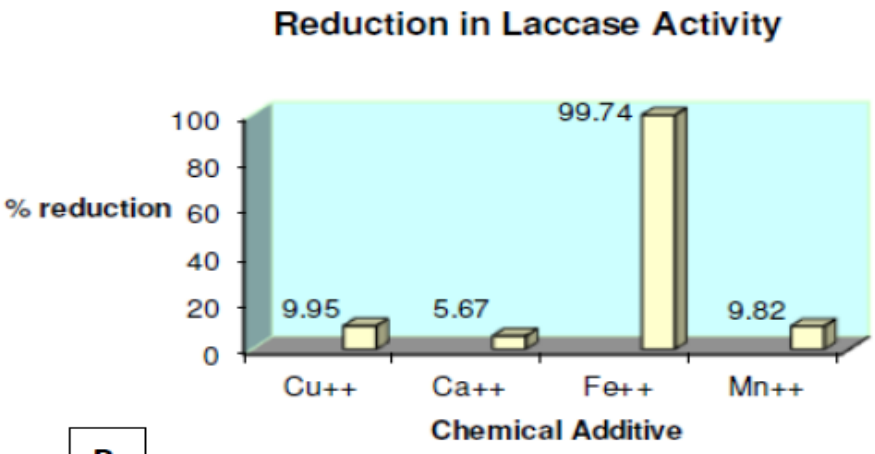
Table 2. Data of Laccase activity after week 2 of fermentation.

WEEK 3	U/mL	Specific Activity
SOLID FERMENTATION: OYSTER		
Corn	2189.72	339.49147
Sorghum	10702.78	718.30738
Sweet potato	2172.22	319.44412
Wheat	21950.00	755.59380
GANODERMA		
Corn	165.28	9.79147
Sorghum	4372.22	354.60016
Sweet potato	597.22	142.19524
Wheat	1358.33	52.24346
SUBMERGED FERMENTATION		
OYSTER		
SUBSTRATE		
Corn	181.39	164.90000
Sorghum	208.89	41.52883
Sweet potato	548.89	135.52840
Wheat	3.54	0.07414
GANODERMA		
Corn	3.82	2.01053
Sorghum	96.11	3.94217
Sweet potato	101.11	26.96267
Wheat	37.85	0.34652

Table 3. Data of Laccase activity after week 3 of fermentation.

WEEK 4	U/mL	Specific Activity
SOLID FERMENTATION: OYSTER		
Corn	713.89	95.18533
Sorghum	8997.22	329.56850
Sweet potato		NA
Wheat	16083.33	345.13584
GANODERMA		
Corn	43.33	2.22205
Sorghum	2111.11	197.30000
Sweet potato	386.11	73.54476
Wheat	1236.11	44.46439
SUBMERGED FERMENTATION		
OYSTER		
SUBSTRATE		
Corn	91.94	37.52653
Sorghum	63.33	13.19375
Sweet potato	383.33	77.44040
Wheat	89.72	1.73205
GANODERMA		
Corn	1.19	0.49583
Sorghum	6.96	1.61860
Sweet potato	57.78	12.69890
Wheat	60.28	0.61826

Table 4. Data of Laccase activity after week 4 of fermentation.



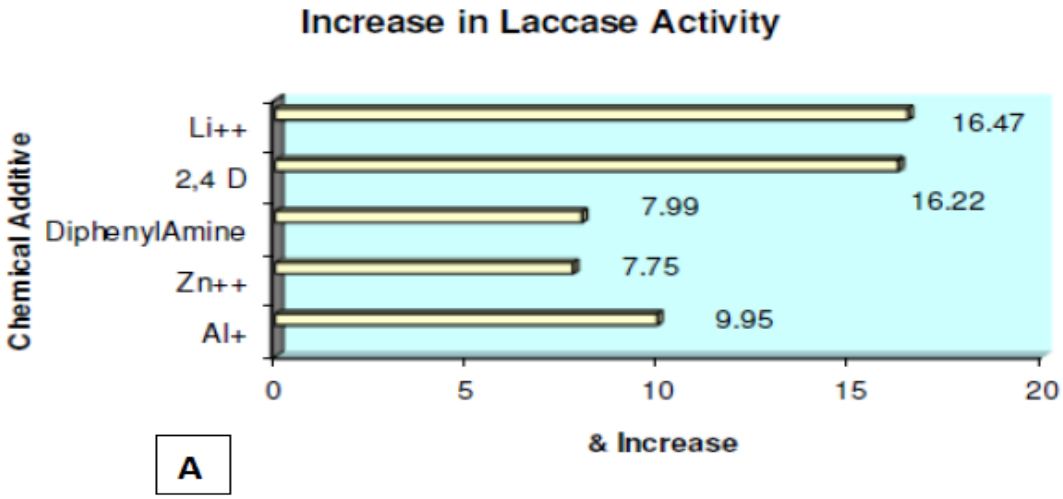


Figure 5. Effect of Chemical Additives on Laccase Activity

Table Analyzed: SOLID VS. LS	Two-way ANOVA , not RM				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	1.451	0.5258	ns	No	
Row Factor	0.07518	0.8843	ns	No	
Column Factor	57.68	0.0014	**	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	139.4	1	139.4	F (1, 12) = 0.4269	P = 0.5258
Row Factor	7.223	1	7.223	F (1, 12) = 0.02212	P = 0.8843
Column Factor	5541	1	5541	F (1, 12) = 16.97	P = 0.0014
Residual	3919	12	326.6		
Number of missing values	0				

Table Analyzed	Two-way ANOVA with RM by columns				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	21.21	0.7697	ns	No	
Row Factor	8.174	0.1383	ns	No	
Column Factor	26.01	0.0266	*	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.937e+008	21	9.223e+006	F (21, 31) = 0.7321	P = 0.7697
Row Factor	7.463e+007	3	2.488e+007	F (3, 31) = 1.975	P = 0.1383
Column Factor	2.375e+008	7	3.393e+007	F (7, 31) = 2.693	P = 0.0266
Residual	3.905e+008	31	1.260e+007		
Number of missing values	1				

Fig.6. Two-way ANOVA analyses

PARTIAL PURIFICATION OF LACCASE USING AMMONIUM SULFATE

% Saturation Ammonium Sulfate	Laccase U/ml	Product Recovery %	Purification Factor (Specific Activity factor)
40%	55.82	15.00%	
50%	1169.44	99.61%	3.19 x
70%	1806.94	99.75%	3.14 x
80%	2191.67	99.80%	3.05 x
90%	1906.67	99.79%	

Table 4A. Partial purification of laccase with ammonium sulfate fractionation.

SUMMARY AND CONCLUSION

Two mushroom cultures, Oyster and Ganoderma were grown in four agricultural substrates (corn, sorghum, sweet potato, and wheat) for optimal growth selection using simple media consisting the substrate and dextrose. Maximum increase in activity of 445x was observed with Oyster grown in solid cultures after three weeks. The highest activity obtained at 3 weeks for Oyster cultures in solid media were at 21K U/ml with wheat: at a specific activity of 756 U/mg. Oyster submerged cultures in bioreactor jars produced stable activities at 3rd week for corn, sorghum and peak activity with sweet potato. Ganoderma cultures in submerged fermentation with sweet potato produced peak activity after 3 weeks, while solid cultures produced peak activities with sorghum and secondly with wheat. Laccase is inhibited by ferrous ions; only minimally with Mn, Cu, and Ca, but enhanced with Li, 2,4 D, Al, Zn, and diphenylamine. Partial purification with 50%, 70%, 80% ammonium sulfate resulted in more than 3x fold increase in specific activity. Product recovery with course filtration after ammonium sulfate precipitation resulted in high recovery rates of more than 99%.

Thus, substantial laccase enzyme activity could be produced with Oyster cultures (although less with Ganoderma cultures by about 1/5) by utilizing simple media with the only use of agricultural crops at a monthly basis.

TECHNO-ECONOMIC DATA

Enzymes contributing to green industrial production strategies like laccases will be replacing chemicals in various industrial processes for sustainable production.

SOCIO-ECONOMIC DATA

Sustainable production using renewable resource base could account for a significant source of livelihood by providing incentives to support the green economy.

RECOMMENDATION

Laccase enzymes is a green technology and have a diverse application in production processes such as in various industries of the food sectors, paper & pulp and textile and environmental bioremediation. Green production line could provide a means to overcome barriers imposed by pollution hazard as these are co-located in highly urban centers.

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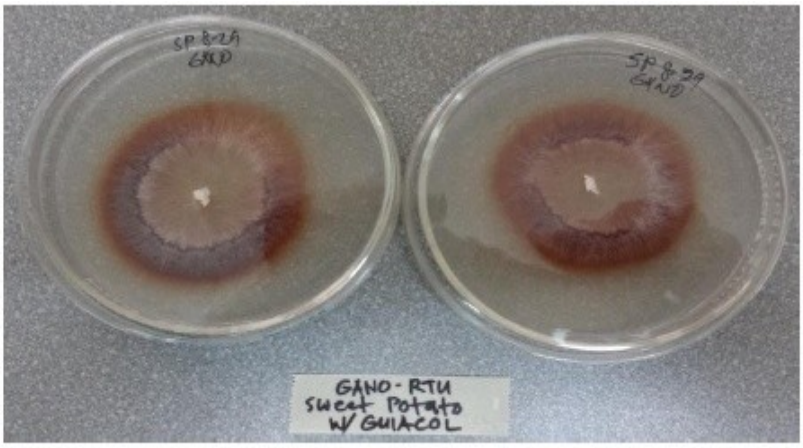
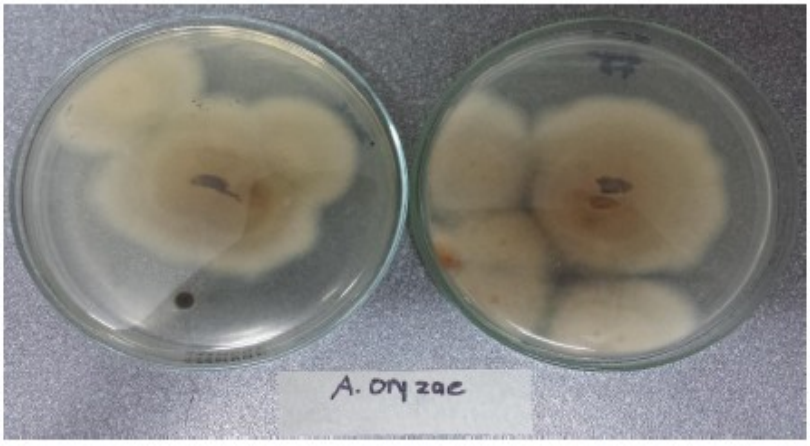
LIST OF TABLES AND FIGURES

Figure 7. METHODOLOGY
Screening for Laccase activity
Inoculation
Solid Substrate Fermentation
Liquid Submerged Fermentation



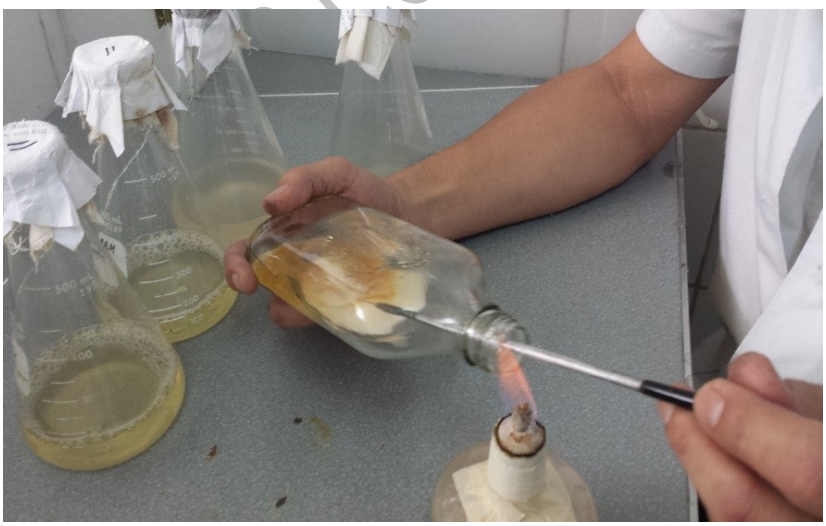
A

Screening for Laccase positive

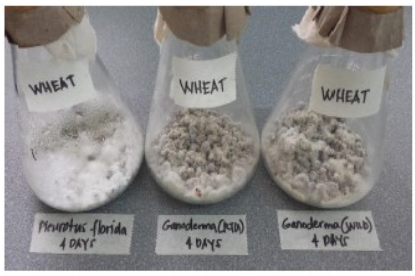


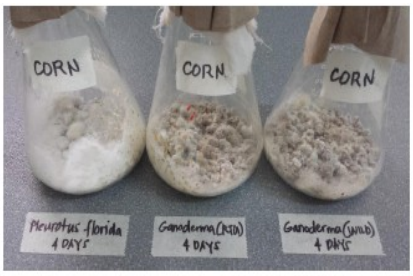
B

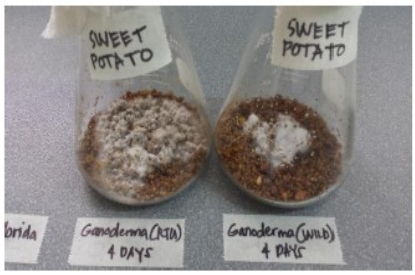
Screening for Laccase negative, control

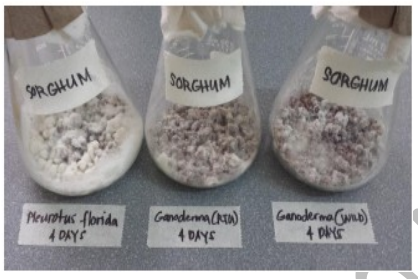


Inoculation





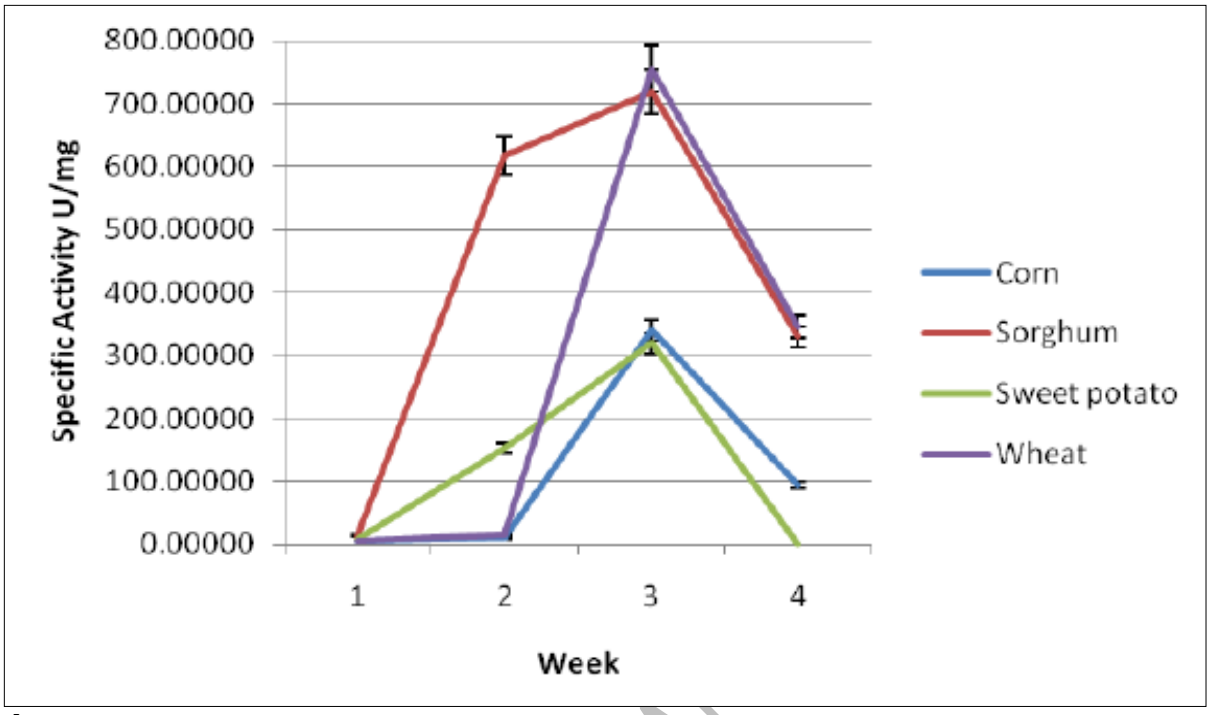




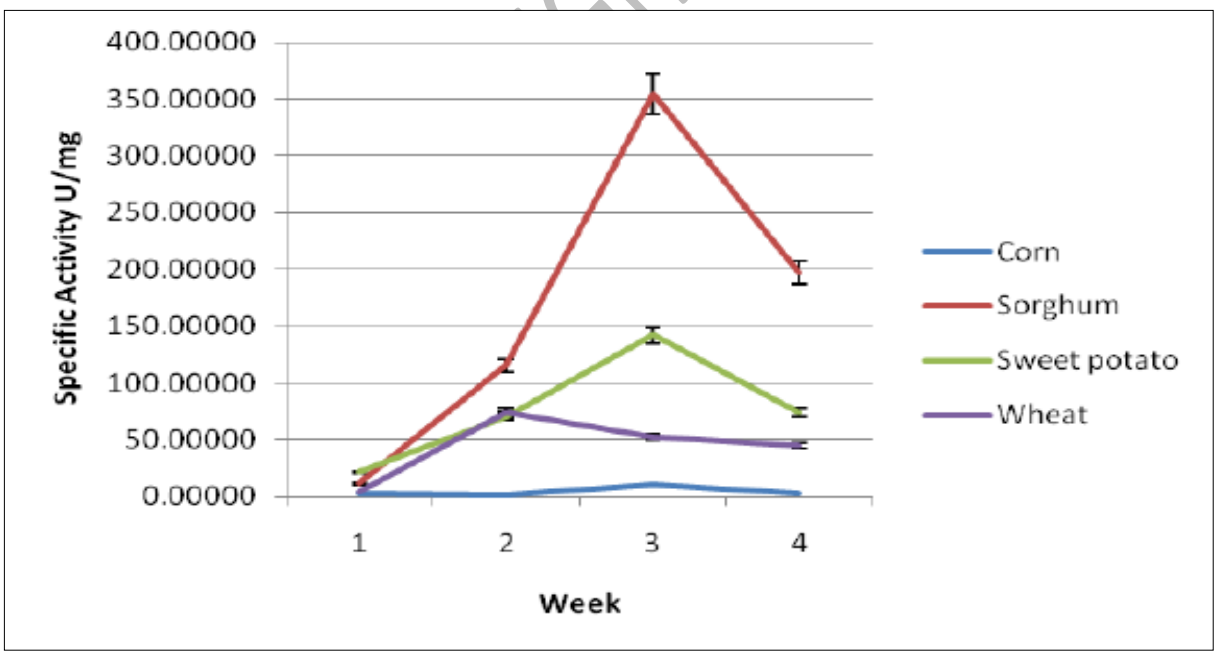
Solid Substrate Fermentation

Liquid Submerged Fermentation

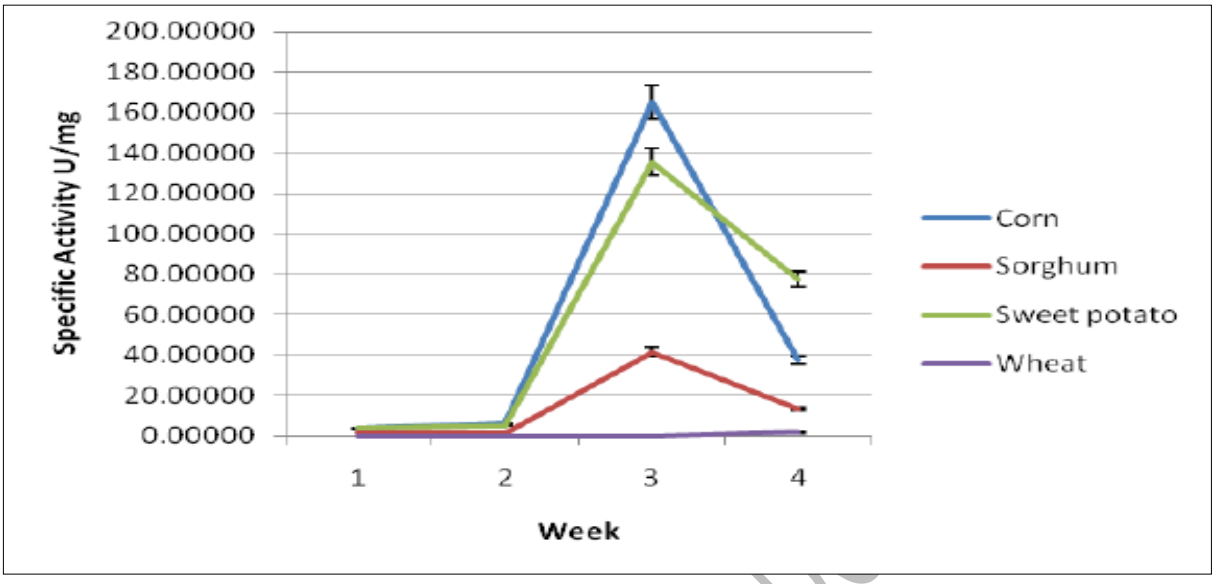
Figure 8. Time Course of Laccase Production. A & C: Oyster cultures while B & D: *Ganoderma lucidum* cultures; A & B: Solid cultures while C & D: submerged cultures.


800.00000
700.00000
600.00000
500.00000
400.00000
300.00000
200.00000
100.00000
0.00000
Specific Activity U/mg
1
2
3
4
Week
Corn
Sorghum
Sweet potato
Wheat

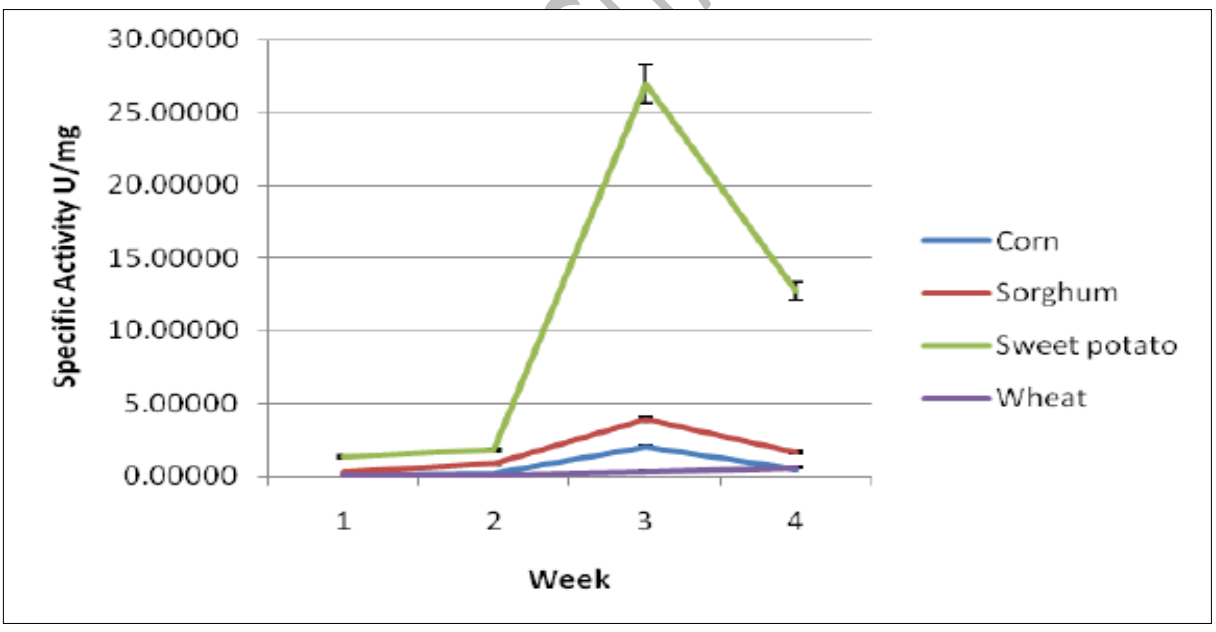
A


400.00000
350.00000
300.00000
250.00000
200.00000
150.00000
100.00000
50.00000
0.00000
Specific Activity U/mg
1
2
3
4
Week
Corn
Sorghum
Sweet potato
Wheat

B


200.00000
180.00000
160.00000
140.00000
120.00000
100.00000
80.00000
60.00000
40.00000
20.00000
0.00000
Specific Activity U/mg
1
2
3
4
Week
Corn
Sorghum
Sweet potato
Wheat

C


30.00000
25.00000
20.00000
15.00000
10.00000
5.00000
0.00000
Specific Activity U/mg
1
2
3
4
Week
Corn
Sorghum
Sweet potato
Wheat

D

Partial Purification of Laccase						
% Saturation (NH4)Sulfate	U/ml	Specific Activity	Protein	% Recovered	Purification Fold (Sp.Activ factor)	Product Enrichment (x Culture factor)
40%	55.82	22.784	2.450	15.00%	0.34	142.40
50%	1169.44	212.625	5.500	99.61%	3.19	1,419.55
70%	1806.94	208.895	8.650	99.75%	3.14	1,397.30
80%	2191.67	202.932	10.800	99.80%	3.05	1,357.25
90%	1906.67	163.663	11.650	99.79%	2.46	1,094.70
Control	873.61	66.561	13.125			

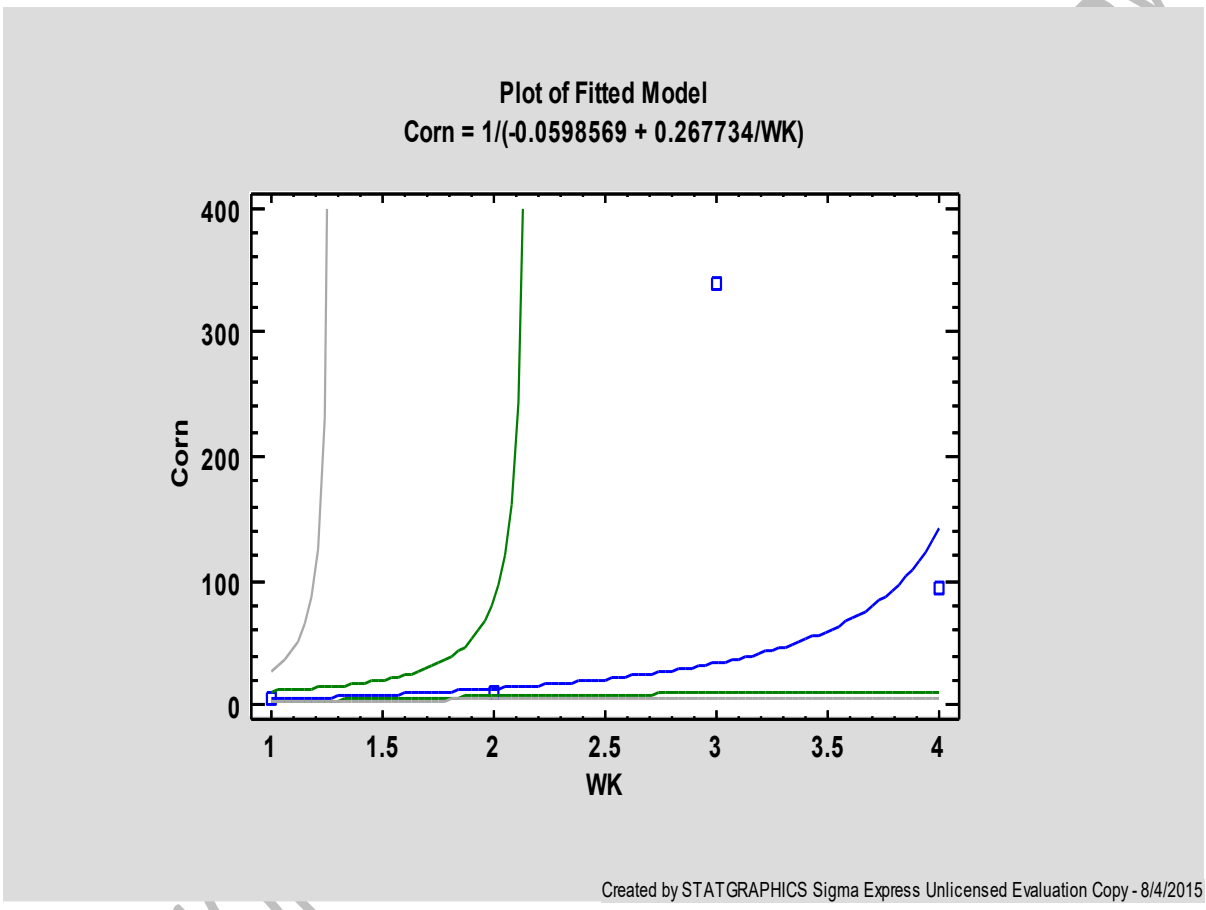
Table 4B. Partial purification of laccase with ammonium sulfate fractionation

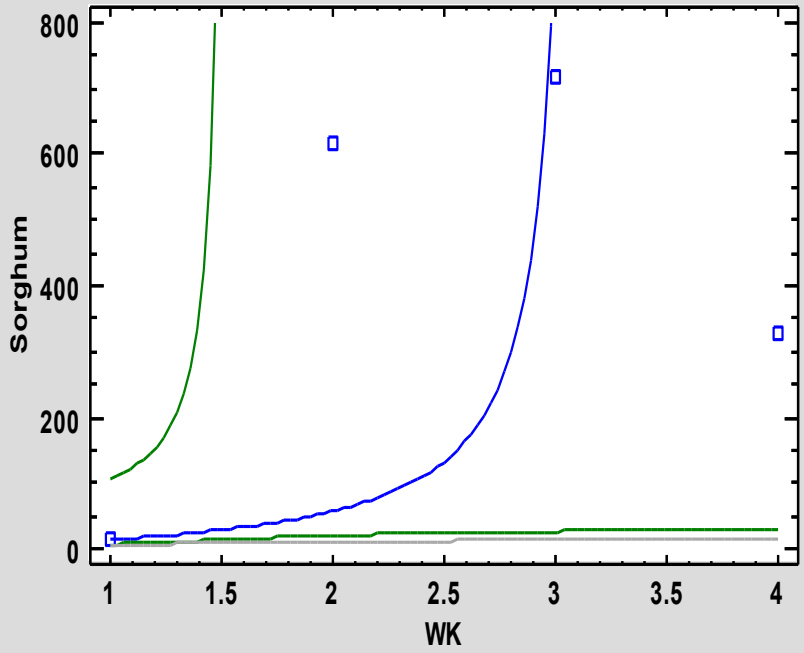
Table Analyzed: SOLID VS. LS	Two-way ANOVA , not RM				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	1.451	0.5258	ns	No	
Row Factor	0.07518	0.8843	ns	No	
Column Factor	57.68	0.0014	**	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	139.4	1	139.4	F (1, 12) = 0.4269	P = 0.5258
Row Factor	7.223	1	7.223	F (1, 12) = 0.02212	P = 0.8843
Column Factor	5541	1	5541	F (1, 12) = 16.97	P = 0.0014
Residual	3919	12	326.6		
Number of missing values	0				

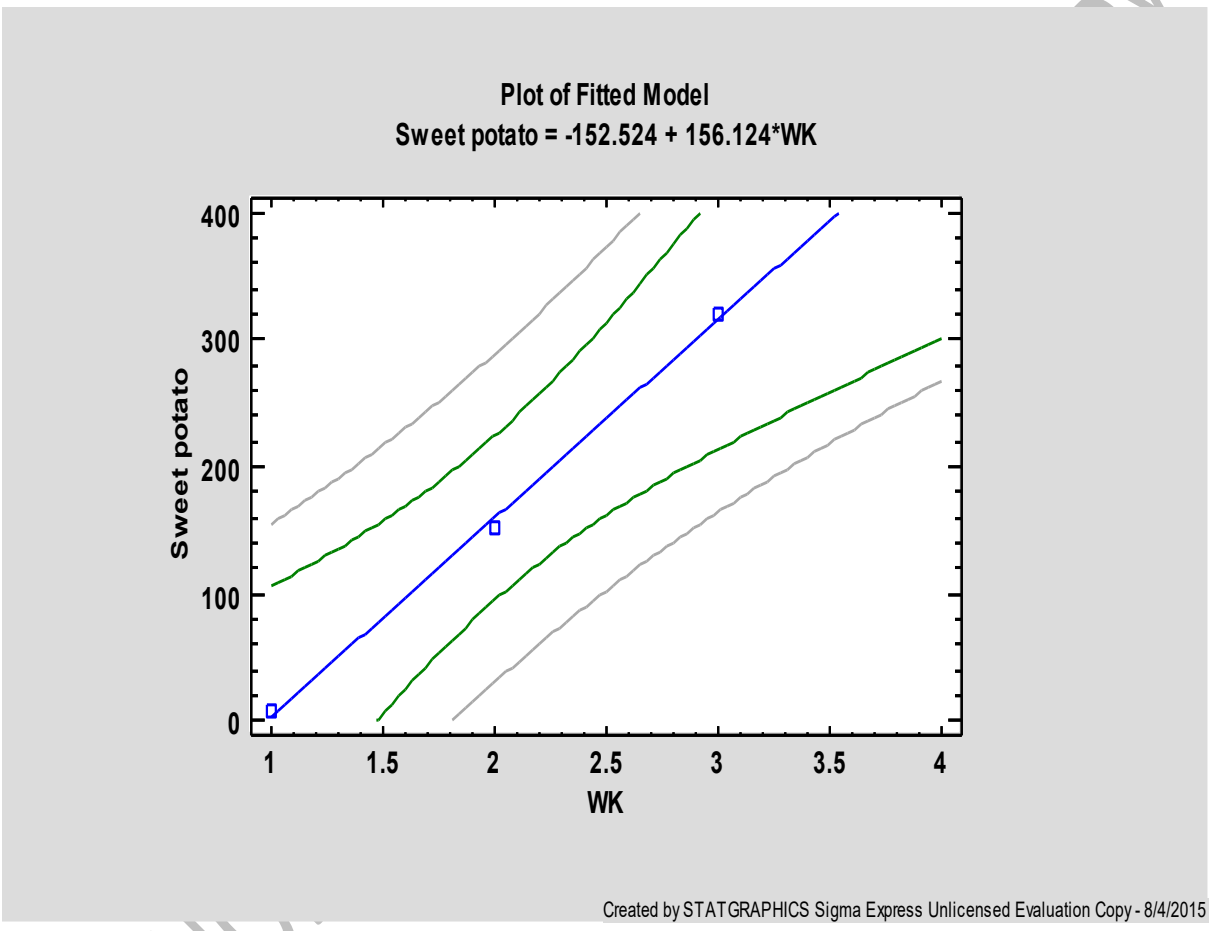
Table Analyzed	Two-way ANOVA with RM by columns				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	21.21	0.7697	ns	No	
Row Factor	8.174	0.1383	ns	No	
Column Factor	26.01	0.0266	*	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.937e+008	21	9.223e+006	F (21, 31) = 0.7321	P = 0.7697
Row Factor	7.463e+007	3	2.488e+007	F (3, 31) = 1.975	P = 0.1383
Column Factor	2.375e+008	7	3.393e+007	F (7, 31) = 2.693	P = 0.0266
Residual	3.905e+008	31	1.260e+007		
Number of missing values	1				

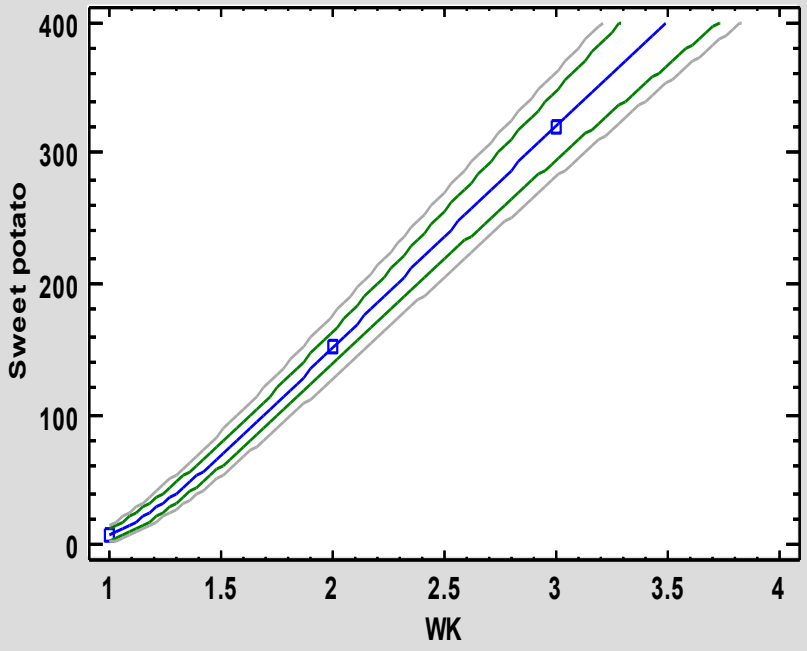
Two-way ANOVA analyses

Figure 9. Data Models of Laccase enzyme yield (U/mg) using agricultural substrates Corn (A), Sorghum (B), Sweet Potato (C,D) and Wheat (E) for Solid Substrate Fermentation (SSF).


Plot of Fitted Model
Corn = 1/(-0.0598569 + 0.267734/WK)
400
300
200
100
0
Corn
1
1.5
2
2.5
3
3.5
4
WK
Created by STATGRAPHICS Sigma Express Unlicensed Evaluation Copy - 8/4/2015


Plot of Fitted Model
Sorghum = 1/(-0.0312046 + 0.0967415/WK)
800
600
400
200
0
Sorghum
1
1.5
2
2.5
3
3.5
4
WK
Created by STATGRAPHICS Sigma Express Unlicensed Evaluation Copy - 8/4/2015


Plot of Fitted Model
Sweet potato = -152.524 + 156.124*WK
Sweet potato
400
300
200
100
0
1
1.5
2
2.5
3
3.5
4
WK
Created by STATGRAPHICS Sigma Express Unlicensed Evaluation Copy - 8/4/2015


Plot of Fitted Model
Sweet potato = (2.70275 + 13.8398*ln(WK))^2
400
300
200
100
0
Sweet potato
1
1.5
2
2.5
3
3.5
4
WK
Created by STATGRAPHICS Sigma Express Unlicensed Evaluation Copy - 8/4/2015

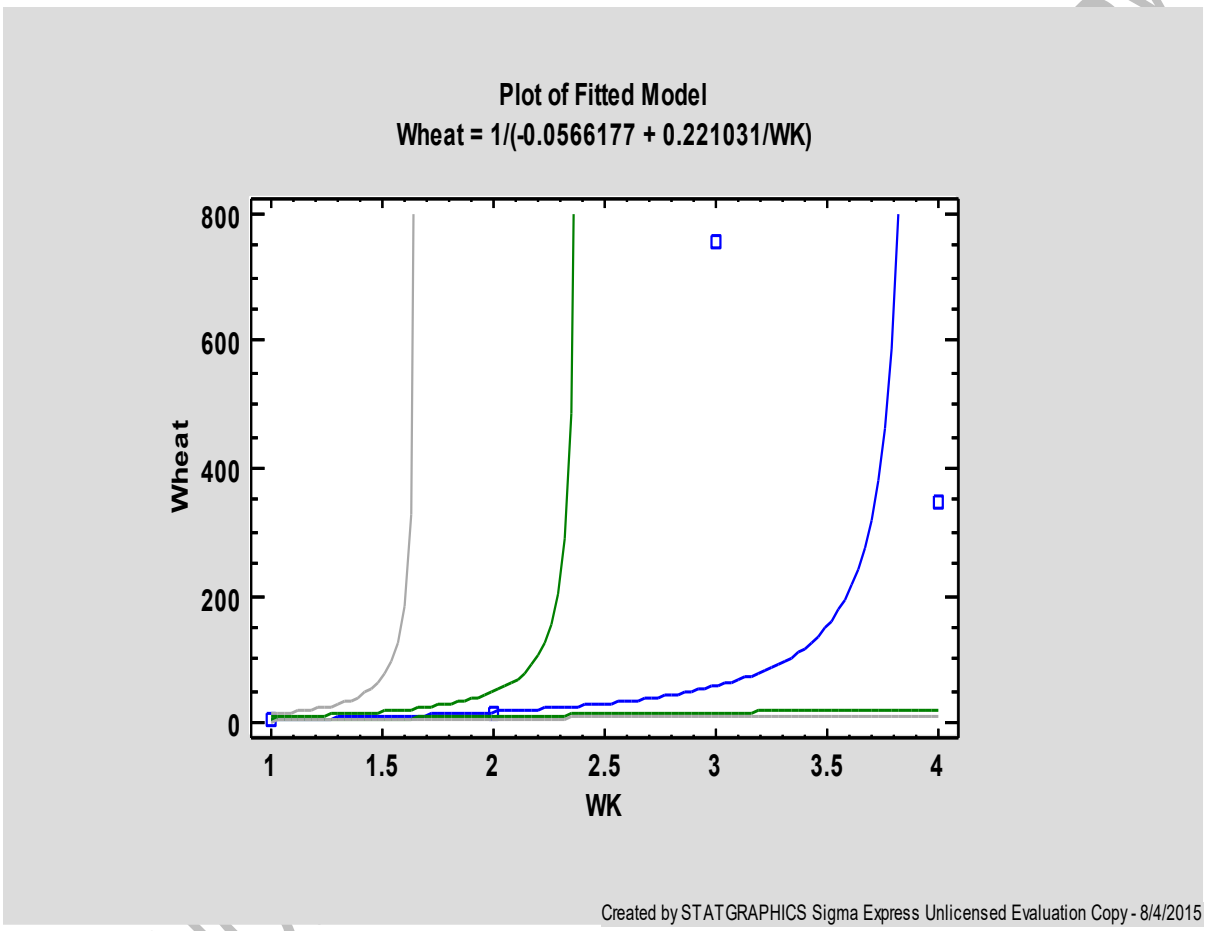
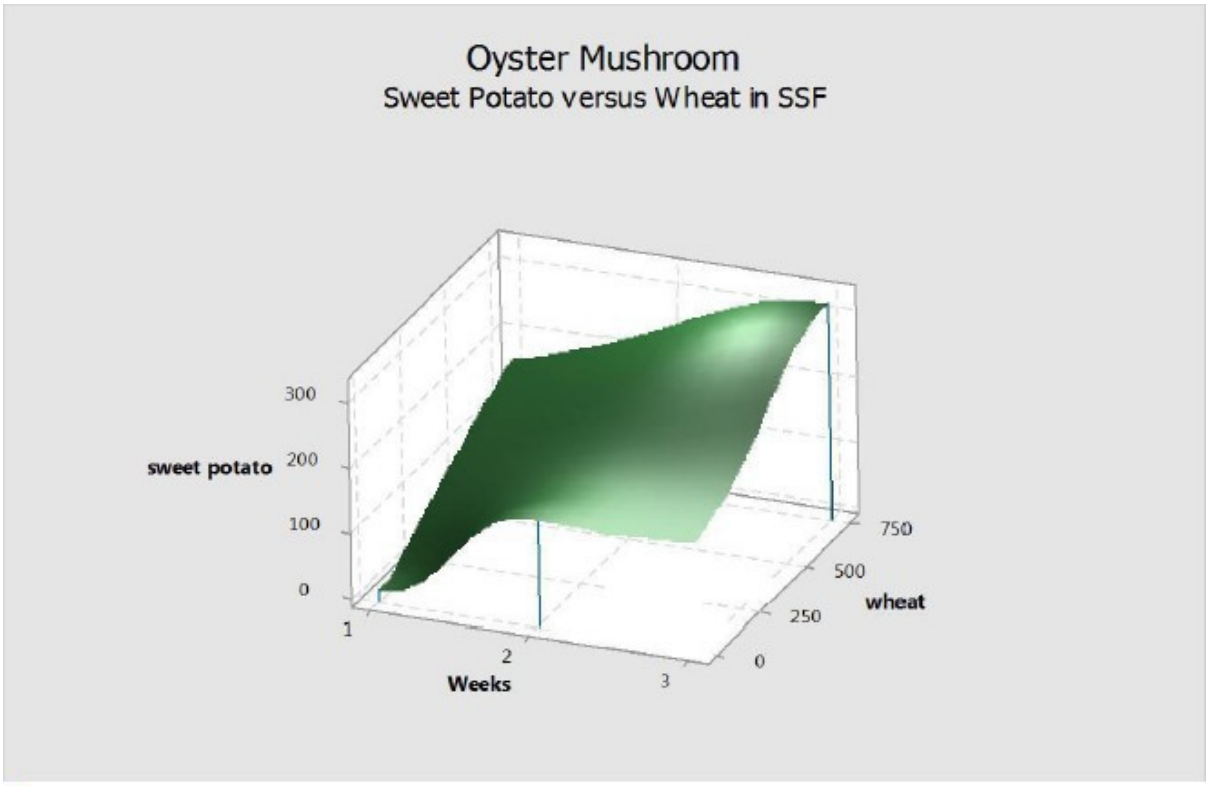

Plot of Fitted Model
Wheat = 1/(-0.0566177 + 0.221031/WK)
800
600
400
200
0
Wheat
1
1.5
2
2.5
3
3.5
4
WK
Created by STATGRAPHICS Sigma Express Unlicensed Evaluation Copy - 8/4/2015

Table 5.

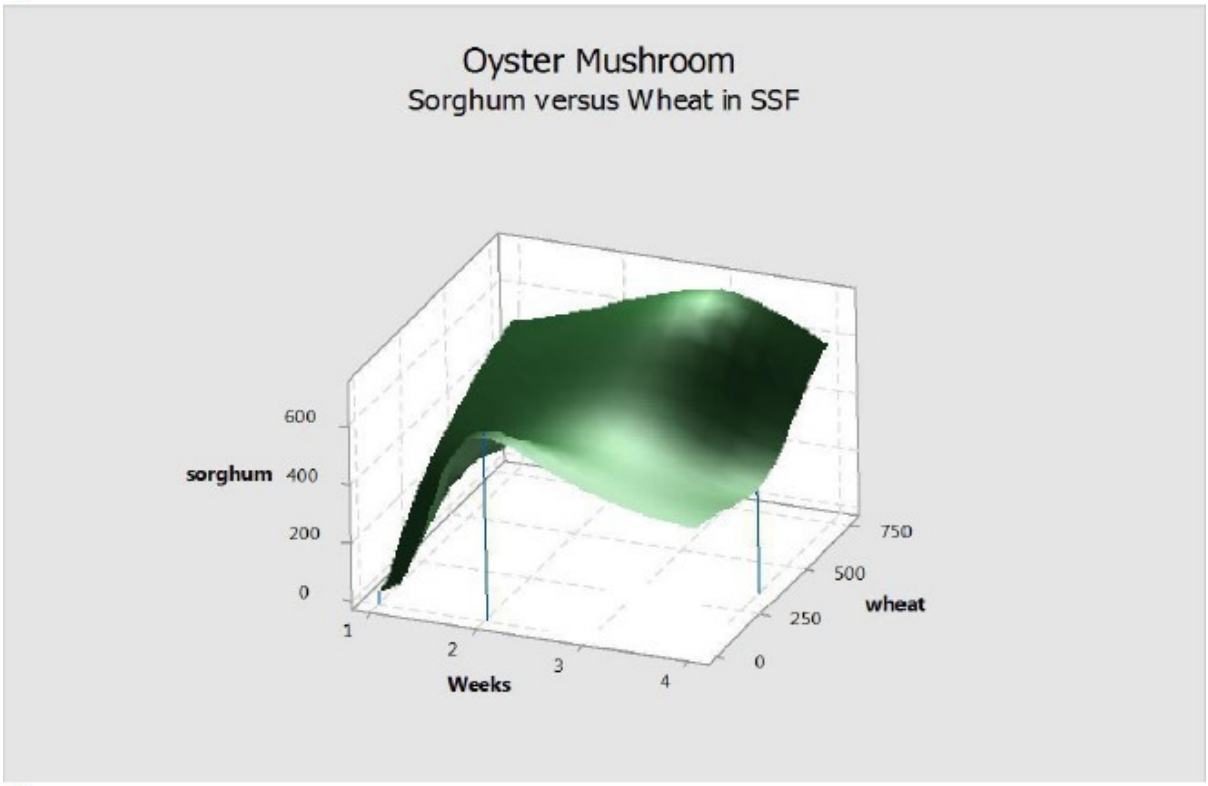
Comparison of Best-Fit Models for Laccase Using Agricultural Substrates for Solid Substrate Fermentation (SSF)

LABEL	Agricultural Substrate Utilized	Model	Correlation	R-Squared	ANOVA p-value	Least-Squares Estimate			
						Intercept	p-value	Slope	p-value
A	CORN	Double reciprocal	0.9673	93.57%	0.0327	-0.0598569	0.1806	0.267734	0.0327
B	SORGHUM	Double reciprocal	0.9464	89.56%	0.0536	-0.0312046	0.1545	0.0967415	0.0536
E	WHEAT	Double reciprocal	0.9856	97.13%	0.0144	-0.0566177	0.0716	0.221031	0.0144
C	SWEET POTATO	Linear	0.9992	99.84%	0.0254	-152.524	0.0560	156.124	0.0254
D	SWEET POTATO	Square root-Y logarithmic-X	1.0000	100.00%	0.0039	2.70275	0.0152	13.8398	0.0039

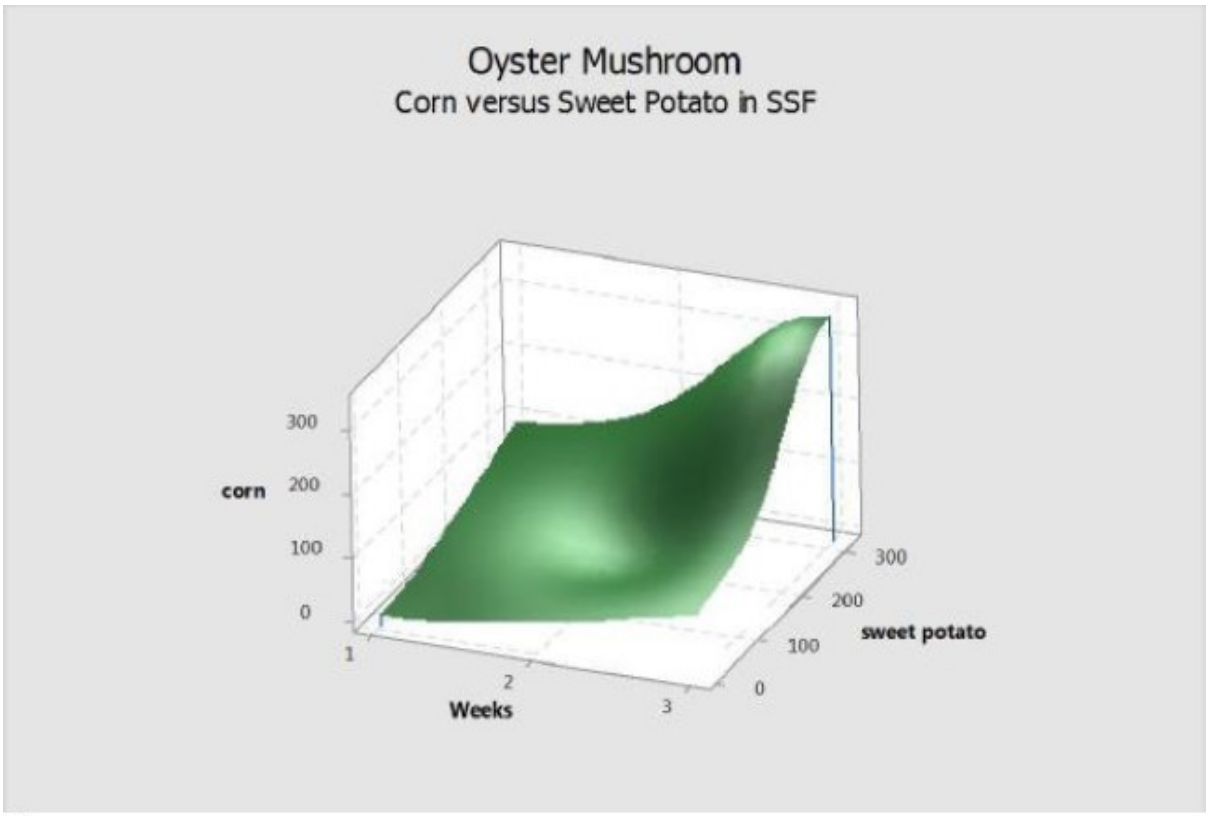
Figure 10. Surface plots of wheat (A&B) and corn interactions (C&D) in SSF.


Oyster Mushroom
Sweet Potato versus Wheat in SSF
sweet potato
300
200
100
0
1
2
3
Weeks
0
250
500
750
wheat

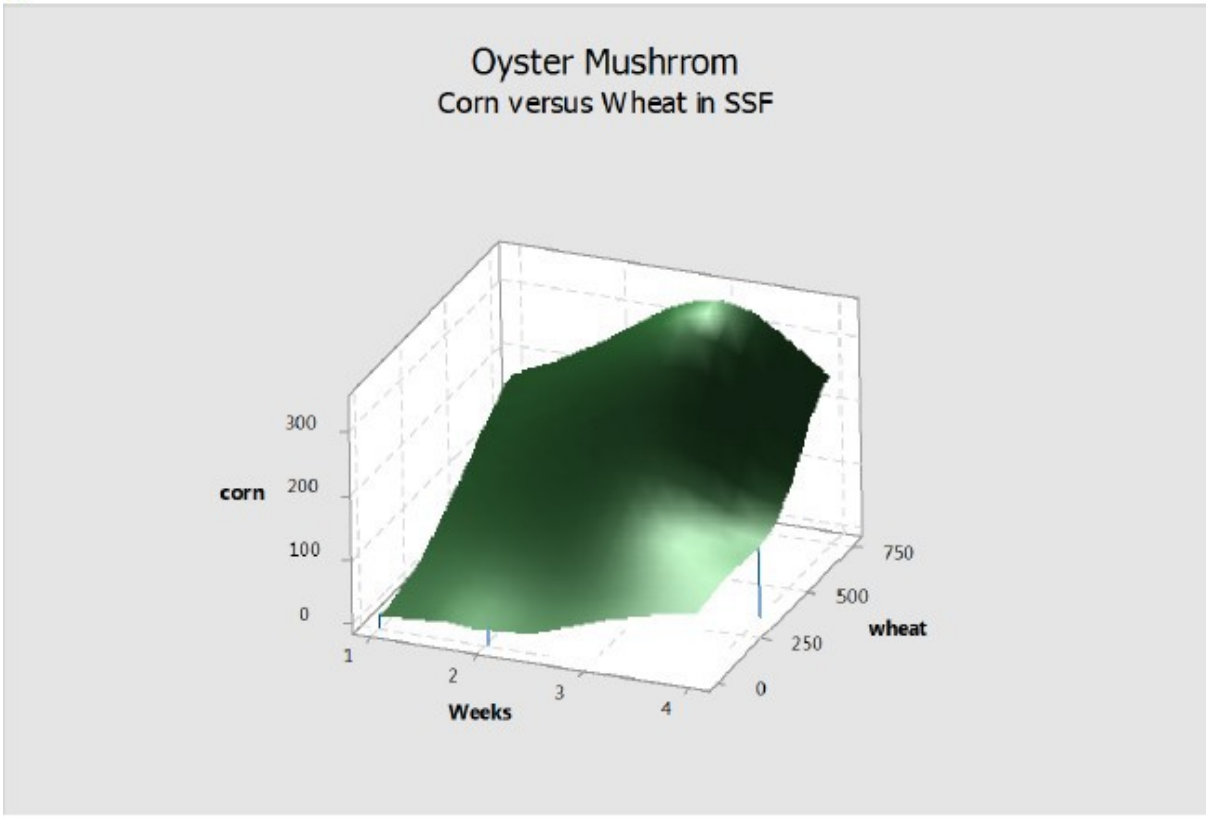
A


Oyster Mushroom
Sorghum versus Wheat in SSF
sorghum
600
400
200
0
1
2
3
4
Weeks
0
250
500
750
wheat

B


Oyster Mushroom
Corn versus Sweet Potato in SSF
corn
300
200
100
0
1
2
3
Weeks
0
100
200
300
sweet potato

C


Oyster Mushrrom
Corn versus Wheat in SSF
corn
300
200
100
0
1
2
3
4
Weeks
0
250
500
750
wheat

D